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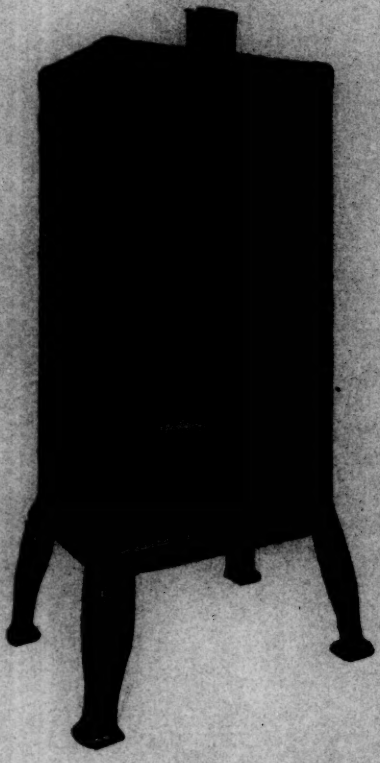

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DETERMINATION OF AMMONIA IN THE BLOOD.

By SERGIUS MORGULIS and H. M. JAHR.

(From the Department of Biochemistry and Physiology, College of Medicine, Creighton University, Omaha.)

(Received for publication, May 1, 1919.)

The estimation of ammonia in the blood is a difficult matter not only because ammonia is present in very minute quantities, but also because it is rapidly split off from some labile components of shed blood. The removal of the ammonia by means of an air current is subject to a source of error in as much as the amount of ammonia recovered may vary with the rapidity of the aeration and the temperature.[1] Besides, the color produced by Nesslerization is too weak to be compared in the colorimeter unless the apparatus is specially adjusted for the purpose,[2] while the microtitration of Barnett[3] requires considerable manipulation which makes the method somewhat cumbersome.

The method which we developed in this laboratory (with the view of future application to the study of some problems) seeks to avoid these disadvantages. In the first place, the blood proteins are precipitated as soon as the blood is taken to prevent the splitting off of ammonia. Secondly, the ammonia in the blood filtrate is absorbed with the aid of permutit[4] which, of course, does away with the necessity of driving off the ammonia by aeration. Furthermore, by the addition of a measured quantity of ammonia to the filtrate, it is possible to examine the color produced by the Nesslerization without changing the colorimeter.

Before this procedure was adopted for the determination of ammonia in the blood, we performed many tests with solutions of albumin in Ringer's solution to which standard ammonium sulfate

[1] Henriques, V., and Christiansen, E., *Biochem. Z.*, 1917, lxxx, 297.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 532.

[3] Barnett, G. D., *J. Biol. Chem.*, 1917, xxix, 459.

[4] Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

had been added to produce a concentration of ammonia similar to that in normal blood. The albumin was precipitated with a 25 per cent solution of *m*-phosphoric acid. To the water-clear filtrate we then added 0.5 mg. of ammonia and sodium hydroxide nearly to neutralize the excess of acid. The mixture was well shaken with several grams of permutit. This was allowed to settle and, after decanting the supernatant fluid, it was washed twice with ammonia-free distilled water. In the meantime a control consisting of an equal volume of Ringer's solution to which was added the same amount of *m*-phosphoric acid and sodium hydroxide to leave the mixture slightly acid, and also containing 0.5 mg. of ammonia, was treated in a similar manner with permutit. The washed permutit of the control and unknown was transferred to 100 cc. volumetric flasks, the absorbed ammonia set free with 1 cc. of 10 per cent sodium hydroxide, and Nesslerized with 15 cc. of Folin-Nessler's reagent. Both flasks were then filled up to the mark, and the color compared in a Duboscq colorimeter. The difference in intensity of the produced color was due to the ammonia present in the original albumin solution. In all our experiments with the artificial "blood," the ammonia recovered by the above method was invariably in accord with the theoretical expectation. These preliminary experiments convinced us, therefore, that the procedure may be properly applied to the determination of the ammonia in the blood.

All the determinations recorded below were made on human blood. This was drawn from the vein of the arm into a large luer syringe containing a little powdered potassium oxalate, and immediately transferred to a 250 cc. volumetric flask. The flask contained approximately 200 cc. of ammonia-free distilled water, and 10 cc. of *m*-phosphoric acid, and was carefully weighed. It was weighed again as soon as the blood was put into it, and enough 25 per cent *m*-phosphoric acid added to have 1 cc. of acid for every 2 gm. of blood taken. The flask was filled to the mark with ammonia-free water and the contents well mixed. It was then allowed to stand for several hours. Duplicate analyses with sheep's blood showed that the length of time during which the material was allowed to stand had no influence upon the amount of ammonia recovered. Generally we let the flasks stand over night because the blood was obtained late in the

afternoon, but sometimes when a sample was gotten in the morning, the contents were filtered and analyzed within 2 or 3 hours. If the material is filtered sooner the filtrate is usually colored and turbid.

The precipitated material was filtered through a folded paper into a graduated cylinder. Generally 200 cc. of a perfectly clear and practically colorless fluid could be collected within a few minutes. This was transferred to a large Erlenmeyer flask and the excess of *m*-phosphoric acid neutralized with 10 per cent sodium hydroxide (sp. gr. 1.12). We found from experience that it required 1 cc. of the hydroxide for every 4 cc. of acid used in the precipitation to reduce the acidity of the mixture to a degree where it was just barely sufficient to redden litmus paper.

The control was made up with 200 cc. of Ringer's solution to which the same quantity of *m*-phosphoric acid and alkali were added. We found difficulty in matching the colors exactly unless the control was treated in just this manner. 5 cc. of the standard ammonium sulfate solution, equivalent to 0.5 mg. of ammonia, were then added to the filtrate from the blood and to the control, and the ammonia in each absorbed with 10 gm. of permutit. The permutit was carefully weighed and washed four times with ammonia-free water to remove all fine particles. After such preliminary treatment the permutit would settle to the bottom quickly like sand and leave no silt-like suspension in the liquid. The flasks were shaken by a continuous rotary motion for exactly 3 minutes. The supernatant fluid was then poured off and the sediment of permutit washed twice with ammonia-free distilled water. The permutit was transferred quantitatively to a 100 cc. volumetric flask, and Nesslerized in the manner already described.

In liberating the ammonia from the permutit, we found it more desirable to use only 1 cc. of the sodium hydroxide, which experiments have shown us to be sufficient to set the ammonia free quantitatively. With this smaller quantity of sodium hydroxide, we practically never had any trouble from the development of turbidity upon Nesslerization.

The following represents a series of determinations of ammonia in the blood of normal individuals:

No.	Subject.	Age.	Quantity of blood.	NH_3 per 100 gm. of blood.
		yrs.	*gm.*	*mg.*
1	Man.	27	21.8	0.30
2	"	41	22.0	0.27
3	Girl.	19	24.0	0.14
4	Man, colored.	32	25.0	0.16
5	Girl.	21	24.0	0.21
6	Man.	60	22.0	0.18

A NOTE ON THE DETERMINATION OF THE INORGANIC CONSTITUENTS OF BLOOD AND OTHER PHYSIOLOGICAL MATERIAL.

By ISIDOR GREENWALD.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York.)

(Received for publication, May 14, 1919.)

In the determination of the inorganic constituents of physiological material it has been usual to destroy completely the organic constituents either by ashing or by treatment with sulfuric and nitric acids. Each of these has its advantages and its disadvantages. There are some objections that are common to both. The oxidation of large quantities of organic substances is troublesome and frequently involves danger both of loss of the substance to be determined and of too great addition of other substances that may interfere with the determination. Any procedure that would reduce the amount of substance to be oxidized, or make this step unnecessary, would seem to be desirable.

Moreover, certain elements such as phosphorus are present both in organic and inorganic combination. It is desirable to be able to distinguish between them. For this purpose it is advisable to avoid high temperatures, as well as high concentrations of either acid or alkali.

The chief organic constituents of animal material are proteins and fats (including lipins). Coagulation by heat has been extensively used to remove the proteins and, with them, the fats. It is very simple but involves the danger of decomposition of organic compounds of the element in question. Moreover, a certain amount of the protein escapes coagulation and frequently interferes with the subsequent procedure.

The author has used for the determination of phosphates,[1] sulfates, calcium, sodium, and potassium[2] a method which he

[1] Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 369; 1915, xxi, 29; 1916, xxv, 431; *Am. J. Med. Sc.*, 1914, cxlvii, 225; *J. Pharm. Exp. Therap.*, 1915, vii, 57.

[2] Greenwald, I., *J. Pharm. Exp. Therap.*, 1918, xi, 281.

believes to be of wide applicability. Because it has been described only incidentally in the presentation of the results obtained with it, it does not seem to have been used by others as much as it otherwise might have been.

The method depends upon the precipitation of the protein and fats by means of picric acid. The exact procedure may vary with the nature of the determination. With blood, it is advisable to dilute to almost 10 volumes with water, add 1 per cent of acetic, hydrochloric, or sulfuric acid, saturate with picric acid, and then dilute to an exact multiple. After standing a few minutes, the mixture is ready for filtering and measured portions are taken for the analyses. The liquid is protein-free and may be oxidized in less than one-tenth the time required for whole blood. In many cases it is unnecessary to oxidize at all.

The method has been employed by Chapin and Powick,[3] Feigl,[4] and by Meigs, Blatherwick, and Cary[5] for the determination of phosphates, by Van Slyke and Donleavy[6] for the determination of chlorides, and, slightly modified, by Halverson and Bergeim[7] for the determination of calcium.

The values obtained by the author[2] for the sodium, potassium, calcium, and "acid-soluble" phosphorus of dog blood and serum are given in Table I.

TABLE I

Inorganic Constituents of Dog Blood and Serum.

	Na	K	Ca	"Acid-soluble" P.
	mg.	*mg.*	*mg.*	*mg.*
100 gm. of blood	286	26.7	18.4	16.4
100 cc. " "	260	24.0		
100 " " serum	297	16.3	12.0	3.63
100 " " "			19.6	
100 " " "	320	27.3	20.2	3.13
100 " " "	304	15.2	21.6	3.50
100 " " "	330	13.9	14.0	3.64
100 " " "	335	15.8	15.3	2.60

[3] Chapin, R. M., and Powick, W. C., *J. Biol. Chem.*, 1915, xx, 97.

[4] Feigl, J., *Biochem. Z.*, 1917, lxxxi, 380; 1917, lxxxiii, 81, 218; 1917, lxxxiv, 264; 1918, lxxxvi, 395; xcii, 1.

[5] Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, i.

[6] Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii,551.

[7] Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.

THE ABSENCE OF FAT-SOLUBLE A VITAMINE IN CERTAIN DUCTLESS GLANDS.*

By A. D. EMMETT and G. O. LUROS.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

(Received for publication, April 15, 1919.)

Of late a good deal of emphasis has been placed upon the possible value of the fat-soluble A accessory in the treatment of certain diseases such as rickets, pellagra, and xerophthalmia. It is known for example that some of the oils, such as cod liver oil, which are admitted to be of therapeutic value, contain a vitamine or food hormone. In 1909 Stepp (1) concluded from his experimental studies on rats that certain fats or related substances, such as lipins, which are soluble in fat solvents, were essential for maintenance and growth. McCollum and Davis (2) and Osborne and Mendel (3) came to the same conclusion in 1913 when they discovered that butter fat stimulated growth in young rats that had thrived normally for a long time and then had ceased to grow. Osborne and Mendel (4) found that this accessory resided in the butter oil fraction and had no relation to lipoids; that it was soluble in absolute alcohol, and when introduced into the diet cured an eye trouble which later McCollum classed as a form of xerophthalmia (5). McCollum and associates who designated this accessory as fat-soluble A (6) found: that, if it was present in animal tissues, it was removed with the fats when they were dissolved out by the ordinary solvents as ether; but in vegetable tissues (plants, seeds, or leaves), it remained behind when the oils were extracted with ether, benzine, chloroform, or acetone (5, 7,). Hot alcohol, however, removed it from the residue (7, 8).

* Read before the Buffalo meeting, American Chemical Society, April 9, 1919.

The following table gives a current summary of the presence or absence of the fat-soluble A vitamine in food substances, based upon published results.

Present.

(*a*) Animal Source:
- Butter fat (2,3).
- Egg yolk and fat (2,4,9).
- Cod liver oil (9).
- Beef oil (10).
- Oleomargarine (10,11).
- Cod testicles (12).
- Pig kidneys (12,13,14).
- Pig liver and liver oil (14).
- Fat fish and fish oils (15).
- Dried and unsweetened condensed milk (16).

(*b*) Vegetable Source:
- Corn, wheat germ, rye, and oats (13).
- Leaves of plants (5,7,17,18).
- Cotton seed flour and oil (19).
- Flaxseed, millet, and hemp seed (7).
- Soy beans (20).
- Peas (21, 22).
- Bananas (23).

Absent or very little.

(*a*) Animal Source:
- Lard (2,4).
- Pig heart (13,14).

(*b*) Vegetable Source:
- Olive oil (2).
- Almond oil (9).
- Oils from maize, linseed, sunflower, soy beans, and wheat (17).
- Cotton seed oil (12, 17).
- Nut margarine (11).
- Vegetable margarine (11).
- White beans (24).
- Barley (25).
- Potato (26).

From the above list, it will be seen that one of the ductless glands, the cod testicles, has been found to contain the fat-soluble A in the ether extract. In this report we add our findings, made upon the fats from three other ductless glands—pancreas, thymus, and suprarenal.

The glands were ground and extracted with either acetone or benzine. The fats were redissolved in ether and again filtered. They were then incorporated in a diet that had been found to be adequate for growth except for a lack of the fat-soluble A accessory. The deficient diet was first fed to rats in order to bring them down to a plane where they would show definite symptoms of a lack of this vitamine such as loss in weight, poor physical condition, and xerophthalmia. The rats were then fed the modified diet and the results compared with control tests (Chart 1) carried out under the same conditions. 10 per cent of the glandular fat was used.

The weight curves, given in Charts 2, 3, 4, and 5, show definitely that benzine or acetone did not extract from the pancreas, thymus, and suprarenal glands a fat that contained the fat-soluble A vitamine. Therefore, in the use of desiccated products of these glands, it is safe to state that any of the ether-soluble fat which is still present (the major portion being removed before desiccation) does not have any therapeutic value from the standpoint of the fat-soluble A vitamine.

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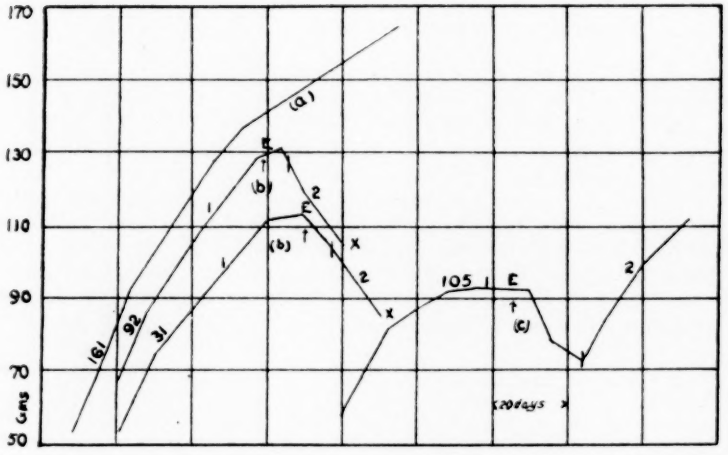

CHART 1. Butter fat. The curves in this chart represent the control groups; (*a*) rats on a diet with fat-soluble A present, (*b*) rats on a diet with fat-soluble A absent, and (*c*) rats that were first put on a deficient fat-soluble A diet, Period 1, and then cured by introducing this accessory into the food mixture, Period 2. "E" designates when xerophthalmia became evident. The basal diet was made up of lactalbumin protein 10 per cent; lard 28 per cent; protein-free milk (the carrier of the water-soluble B vitamine, mineral salts, and lactose) 28 per cent; and starch. 10 per cent of purified butter fat was employed to supply the fat-soluble A and replaced an equivalent amount of lard.

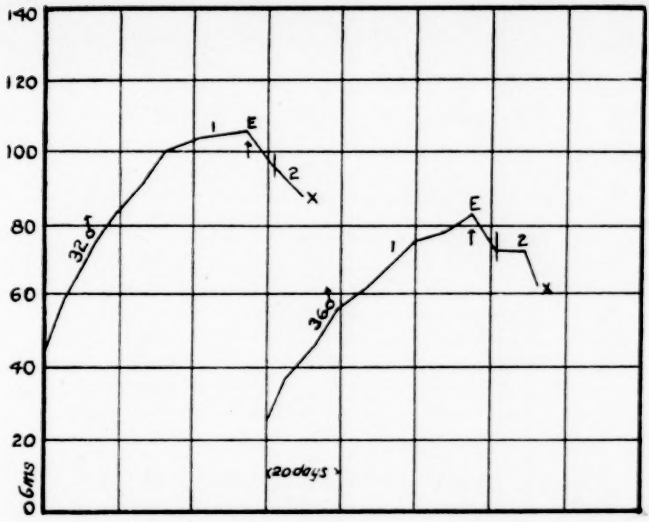

CHART 2. Pancreas fat. In Period 1, the rats were fed the basal diet. Period 2, when the acetone-extracted pancreas fat was used, indicates that the rats did not respond to the change in diet but died as designated by "X."

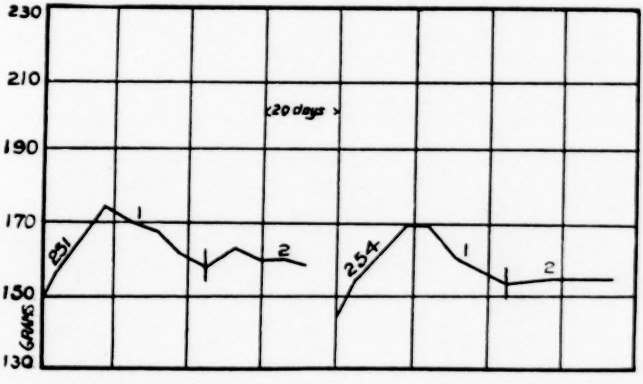

CHART 3. Pancreas fat. In Period 1, the basal diet was used. In Period 2, 10 per cent of the pancreas fat was introduced into the ration. It was obtained by extracting the glands with ether instead of acetone as in Chart 2. These rats were much heavier at the start than those represented in Chart 2. There was a cessation in the loss of weight but no gain in weight and no improvement in bodily condition. The rats finally became so weak that they were killed.

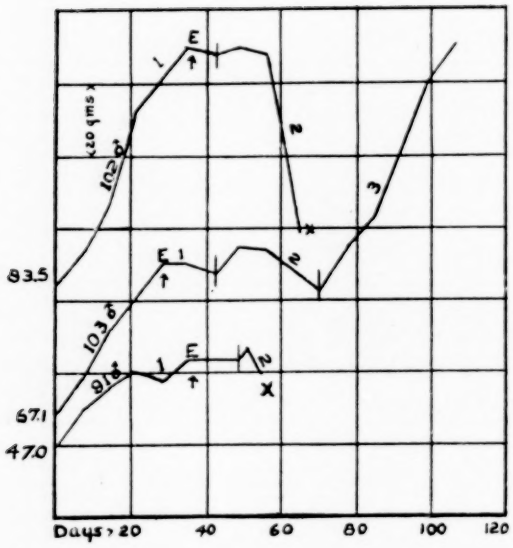

CHART 4. Thymus fat. In Period 1, the diet was the same as the basal food fed the rats represented in Charts 1, 2, and 3. Period 2 shows when the acetone-extracted thymus fat was introduced, there was no beneficial effect resulting, only a continued decline. In Period 3, the equivalent amount of butter fat, substituted for the thymus fat, brought about immediate response.

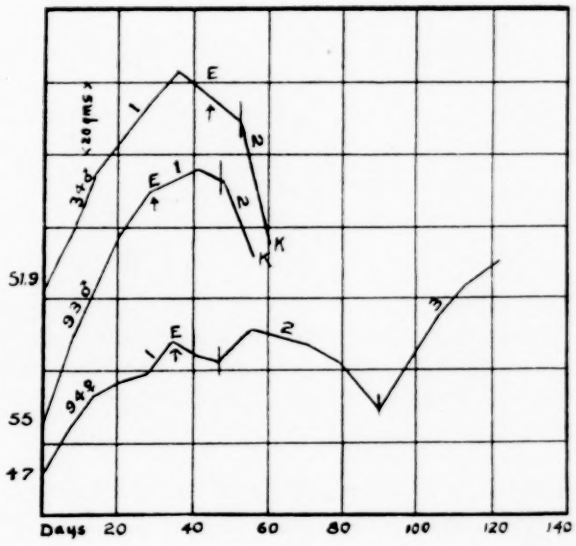

CHART 5. Suprarenal fat. At the end of Period 1, during which the basal diet was fed, 10 per cent of the ether-extracted suprarenal fat was put in the food mixture, in place of so much lard. No growth, gain in weight, or improvement in body took place. Upon introducing 10 per cent of purified butter fat for the suprarenal fat, an immediate response in condition and weight was manifested. Rats 34 and 93 were in such an emaciated condition that they were killed, as shown in the chart by "K."

A METHOD FOR DETERMINATION OF MINUTE AMOUNTS OF LEAD IN URINE, FECES, AND TISSUES.

By W. DENIS and A. S. MINOT.

(From the Chemical Laboratory of the Massachusetts General Hospital, Boston.)

(Received for publication, May 24, 1919.)

There is great need for a relatively simple and rapid method for the quantitative determination of lead in biological material. At present such a method is not available; indeed we have found that the great majority of the processes described in the literature for even the qualitative detection of lead in urine and feces is extremely laborious and unsatisfactory. The method described below is the result of a critical study of the subject which has extended over a period of five years, and in our hands has given excellent results.

Stated briefly, the process consists essentially in the removal of most of the organic matter by fusion with sodium nitrate, the precipitation of the lead as sulfide, its electrolytic deposition as lead peroxide, and the titration of the iodine liberated from potassium iodide when the lead peroxide deposit is treated with dilute acid. The detailed description of the procedure is as follows.

Determination of Lead in Urine.

Evaporate to dryness 2,000 cc. of urine and allow the dry residue to char slightly. When cool mix the residue with 8 gm. of powdered sodium nitrate and transfer to a small silica evaporating dish or crucible. Heat cautiously until the first violence of the reaction is over, and then more strongly, with a Meker burner or in a muffle furnace, until most of the organic matter has been removed. When cool the material in the dish is transferred to a 300 cc. beaker and treated with 10 per cent hydrochloric acid until a faintly acid reaction is obtained. The acid solution is

then heated to boiling, filtered when still very hot, and the residue on the filter washed thoroughly with boiling water. To the filtrate concentrated ammonium hydroxide is added until a faintly alkaline reaction is obtained, and after the addition of two or three drops of a 1.0 per cent solution of copper sulfate the lead and copper are precipitated by means of ammonium sulfide or hydrogen sulfide.

The suspension of sulfides is transferred to a 50 cc. centrifuge tube, and after centrifuging, the supernatant liquid is poured off, and the sediment washed once with water, two to six times with 0.1 N hydrochloric acid, and finally once again with water. The use of the centrifuge for the removal of the precipitated phosphates greatly facilitates the work, when only one or two determinations are being carried on at one time, but when a large number of analyses are being done simultaneously it is generally more convenient to conduct the process by means of decantations from flasks, and to use the centrifuge only when practically all the phosphate precipitate has been removed.

The purpose of this rather elaborate scheme of washing is to remove magnesium, phosphates, and chlorides, all of which interfere with the deposition of lead as peroxide. The use of an acid of a concentration greater than 0.1 N appears to give low results. The final residue which consists of the sulfides of lead and copper is transferred to a platinum crucible of 25 cc. capacity by means of 3 cc. of dilute nitric acid (20 parts concentrated nitric acid, sp. gr. 1.42, and 80 parts water) and the tube washed with 3 cc. of distilled water, which are added to the contents of the crucible. The solution is then electrolyzed with a current of 3 to 3.5 amperes and 5 to 6 volts. The crucible serves as the anode, the cathode consisting of a piece of platinum wire 60 mm. long and 1.0 mm. in diameter which is twisted to form a spiral. In our work we have used a storage battery current having an E.M.F. of 16 volts; in addition to the rheostat, an ammeter and voltmeter are kept in the circuit during the entire period of electrolysis. On account of the small volume of the electrolyte, the temperature rises rapidly and generally reaches a maximum of 75°C. within 2 or 3 minutes after the current is turned on. Within 15 minutes all the lead is deposited on the crucible anode in the form of a brown film of lead peroxide, while the copper and

any traces of iron which may be present have been deposited on the cathode.

If the current is turned off while the lead peroxide is in contact with nitric acid a considerable portion of the deposit is dissolved before it is possible to pour off the acid electrolyte. It is therefore necessary to remove the acid liquid by means of a siphon while the electrolysis is still going on. As the acid is drained off, distilled water is introduced and this process is continued until the marker of the ammeter has dropped to zero, showing the removal of practically all electrolytes.

The crucible is disconnected, washed out once or twice with distilled water, and to it are then added 5 cc. of a 5 per cent solution of potassium iodide (free from iodate) and 1 cc. of 25 per cent acetic acid. The lead peroxide is almost instantly decomposed, giving a deposit of yellow lead iodide on the crucible, and a solution of free iodine. The mixture should be allowed to stand for 5 minutes to allow the reaction to be quantitatively completed, and the liquid is then transferred to a small beaker and the iodine titrated with 0.005 N sodium thiosulfate solution using starch as indicator. 1 cc. of 0.005 N sodium thiosulfate solution is equivalent to 0.517 mg. of lead.

This titration is an adaptation of a method in use for many years in technical laboratories for the assay of hydrogen peroxide preparations.[1]

Determination of Lead in Feces and Tissues.

About 500 gm. of soft feces or half that amount of more solid material, or 100 gm. of muscle, liver, or other tissue are spread in a thin layer on an ordinary china dinner plate, and the moisture rapidly removed by allowing the plate to stand on a gas or electric hot plate. If the layers are made sufficiently thin the process is usually completed within one or at most two hours.

The dry material is then ground to a coarse powder in a porcelain mortar, mixed with one-half its weight of sodium nitrate, transferred to a silica evaporating dish or crucible, and heated

[1] 0.005 N sodium thiosulfate does not keep well and a fresh preparation should be prepared (by dilution of the 0.1 N solution) at intervals of about 10 days.

with a Bunsen burner. Oxidation begins promptly and must, if too violent, be controlled by removing the burner. After the first reaction is over the dish should be strongly heated for 10 to 20 minutes either with a Meker burner or in a muffle furnace. The subsequent treatment is the same as already described for urine. In the case of feces from patients who have received magnesium sulfate, the precipitate obtained when the acid extract of the fusion is made alkaline is extremely heavy and requires, in the more extreme cases, a more prolonged treatment with dilute hydrochloric acid than is described for urine.

Where quantitative results are not desired the above process may be used for the qualitative detection of lead, as the lead peroxide deposit and the film of lead iodide are of characteristic appearance. As little as 0.1 mg. of lead can easily be detected by this means.

When carried out on the quantitative basis we have been able to recover lead added to urine, feces, and tissues in amounts varying from 0.5 to 4 mg. to the average extent of 95 per cent of the amount added. It is of course obvious that only sodium nitrate and nitric acid which have been tested and found free from lead should be used.

When nitric acid is electrolyzed in a platinum crucible and acidified potassium iodide introduced into the crucible (after the latter has been carefully washed with distilled water) a red color is produced, which gives a blue color with iodine, and which is discharged by one or two drops of 0.005 N thiosulfate solution. We have been unable to find out the cause of this phenomenon. Nitric acid twice distilled from a Jena glass retort gives results essentially similar to the ordinary high grade C.P. nitric acid. On account of this coloration qualitative tests for lead made by this method should not be reported "positive" unless the deposit of lead iodide is present. In quantitative work this "blank" should be determined and the amount of thiosulfate used to discharge the color so obtained should be subtracted from the result of the titration.

THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF COW'S MILK.

By W. DENIS and A. S. MINOT.

(From the Chemical Laboratory of the Massachusetts General Hospital, Boston.)

(Received for publication, May 24, 1919.)

But little reliable data is at present available regarding the quantitative relationships of the non-protein nitrogenous constituents of milk.

The qualitative composition of this fraction has been known, to a certain extent at least, for many years. Amino-acids, urea, creatine, creatinine, purine bodies, and ammonia have long been recognized as constituents of this fluid, but on account of the lack of appropriate analytical methods we are still relatively ignorant of the amounts of these bodies present in milk either under normal or under pathological conditions. In a recent paper from this laboratory[1] a description was given of the analytical technique which had been worked out for the quantitative determination of some of the non-protein constituents of milk, and in the present communication we wish to present results obtained by means of these methods on a considerable number of samples of cow's milk.

The results presented in Table I were obtained on the mixed product of four large milk distributors in Boston, and on thirty-eight samples of milk from individual cows which were personally collected by us in three dairies.

An inspection of these results would seem to show that while different samples of mixed milk of a large number of cows give practically uniform results, irrespective of variety of origin, the milk taken from individual cows in different dairies tends to give striking differences in the values for non-protein nitrogen and urea.

[1] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxvii, 353.

TABLE I.

Non-Protein Nitrogenous Constituents of Cow's Milk.

Cow No.	Amount per 100 cc. of milk.						Remarks.
	Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.*	Preformed creatinine.*	Creatine.*	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
A	21.9	10.0	4.03	1.4	1.3	2.3	Mixed milk from milk dealer A.
B	21.0	9.0	4.19	1.6	1.4	2.4	" " " " " B.
C	20.0	9.0	4.50	1.6	1.5	2.4	" " " " " C.
D	19.0	6.8	4.18	1.8	1.0	2.2	" " " " " D.
102	22.0	8.0	5.1	1.6	1.1	2.2	Half Holstein, half Jersey, 2 yrs. old; calf 4 months old.
103	25.0	11.0	3.5	1.4	1.0	2.5	Jersey, 4 yrs. old; calf 6 months old.
104	24.0	6.9	3.2	1.4	1.0	2.5	Jersey, 5 yrs. old; calf 8 months old.
105	25.0	8.4	3.2	1.4	1.1	2.3	Jersey, 2 yrs. old; calf 2 months old.
106	25.0	5.2	3.5	1.5	1.0	2.2	Half Jersey, half Guernsey, 6 yrs. old; calf 6 months old.
107	23.0	8.2	3.2	1.6	1.1	2.2	Jersey, 2 yrs. old; calf 6 months old.
108	22.0	5.4	2.6	2.0	1.1	2.0	Jersey, 6 yrs. old; calf 4 months old.
109	23.0	8.6	4.4	1.6	1.0	2.2	Mixed Jersey and Holstein, 2 yrs. old; calf 4 months old.
110	25.0	7.8	3.6	1.4	1.0	2.5	Mixed Jersey and Holstein, 7 yrs. old; calf 3 months old.
111	—	6.0	—	—	1.1	2.3	Mixed breed, 12 yrs. old, about dry; calf 10 months old.
112	22.0	5.7	4.8	1.3	1.2	2.1	Jersey, 7 yrs. old; calf 3 days old.
116	38.0	20.0	4.9	1.6	1.1	2.2	Holstein, 2 yrs. old; calf 3 months old.
117	32.0	15.0	7.3	1.4	1.1	2.3	Holstein, 3 yrs. old; calf 10 months old.
118	34.0	17.4	5.3	1.8	1.2	2.2	Holstein, 10 yrs. old; calf 2 months old.
119	29.0	15.0	4.7	1.6	1.2	2.3	Holstein, 10 yrs. old; calf 8 months old.
44	28.0	7.8	—	—	1.1	2.3	Holstein.
60	30.2	8.3	—	—	1.1	2.2	"
38	25.7	8.0	—	—	1.2	2.0	Mixed breed.

TABLE I—*Concluded.*

Cow No.	Amount per 100 cc. of milk.						Remarks.
	Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.*	Preformed creatinine.*	Creatine.*	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
34	24.7	10.8	—	—	1.0	2.5	Holstein.
5	27.5	10.0	—	—	1.1	2.2	"
11	25.0	8.3	—	—	1.1	2.3	Mixed breed.
45	27.0	8.7	—	—	1.2	2.2	" "
2	26.9	7.5	—	—	1.2	2.4	Holstein.
47	30.2	8.0	—	—	1.0	2.6	Jersey.
31	29.2	9.0	—	—	1.0	2.4	Mixed breed.
43	28.0	10.8	—	—	1.0	2.5	Holstein.
42	24.5	6.0	—	—	1.0	2.2	"
37	24.7	8.7	—	—	1.0	2.2	"
41	30.2	8.7	—	—	1.1	2.2	"
58	30.0	9.3	—	—	1.2	2.3	"
28	29.0	8.4	—	—	1.2	2.3	Jersey.
59	24.7	10.0	—	—	1.1	2.5	"
57	27.0	8.0	—	—	1.1	2.4	Holstein.
22	26.0	8.8	—	—	1.1	2.3	Jersey.

* The figures for uric acid, creatinine, and creatine indicate the amounts of these substances, not of their nitrogen.

Thus in Samples 102 to 112 inclusive which were obtained from Dairy M. at Bath, New Hampshire, the values for non-protein nitrogen vary from 22 to 25 mg. and the urea nitrogen from 5.2 to 11 mg. per 100 cc. of milk; Samples 116 to 119 inclusive were obtained from cows belonging to the herd of Dairy B., also at Bath, New Hampshire, and here it will be noted that the figures for non-protein nitrogen and urea nitrogen are on a considerably higher level than in the samples obtained from Dairy M. Samples 5 to 60 were obtained from the dairy of the McLean Hospital at Waverley, Massachusetts, and show values intermediate between those given by the cows in the other two herds.

The above results would seem to point to the influence of food intake, more particularly protein intake, on the relative composition of the non-protein fraction of milk.

Through the courtesy of the owners of the two New Hampshire dairies we were enabled to obtain direct experimental evidence

on this point. Three cows were maintained for us during a period of nearly 2 months on weighed rations. The first samples of milk were taken when the cows were on the ration used for routine feeding by their respective owners. They were then placed on a ration differing in a marked degree as regards protein content from that which had been previously fed, the second milk sample being taken after the cows had eaten this food for a period of 30 days.

The results of this experiment are given in Table II, while in Table III are presented the results of a few observations made on seven cows also fed on rations of known composition.

TABLE II.

*Effect of Protein Intake on the Concentration of Some of the Non-Protein Constituents of Milk.**

Cow.	Date.	Weight of cow.	Nutritive ratio of feed.	Digestible protein per day.	Non-protein constituents per 100 cc. of milk.						Remarks.
					Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.	Preformed creatinine.	Creatine.	
		kg.		*kg.*	*mg.*	*mg.*	*mg.*	*mg*	*mg.*	*mg.*	
M	Dec. 29, 1918	300	1:12	0.52	22	—	2.6	2.0	1.1	2.0	Jersey.
M	Feb. 24, 1919	300	1:5.8	0.96	34	19.0	3.1	2.0	1.3	2.1	
B1	Dec. 29, 1918	636	1:5.2	1.12	32	14.8	7.4	1.4	1.1	2.3	Holstein.
B1	Feb. 24, 1919	636	1:8	0.80	22	9.8	7.0	1.4	1.2	2.0	
B2	Dec. 29, 1918	545	1:5.2	1.12	29	15.0	4.8	1.6	1.2	2.1	"
B2	Feb. 24, 1919	545	1:80	0.80	22	8.6	3.3	1.9	1.2	2.0	

* The foods composing the rations of these cows consisted of hay, oats, barley, cottonseed meal, and silage. The amount of digestible protein, the nutritive ratios, and the ratio of digestible protein to other foods (fats and carbohydrates) of the rations were calculated from the tables given by W. A. Henry and F. B. Morrison (Feeds and feedings, Madison, 16th edition, 1916).

The results presented in Tables II and III leave no doubt of the influence of protein intake on the content of non-protein nitrogen, urea, and amino nitrogen in cow's milk. The other non-protein nitrogenous bodies which have been determined are apparently unaffected by the nature of the food.

TABLE III.

Non-Protein Constituents of Milk of Cows on Weighed Rations.

Cow No.	Weight of cow.	Nutritive ratio of food.	Digestible protein per day.	Non-protein constituents per 100 cc. of milk.						Remarks.
				Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.	Preformed creatinine.	Creatine.	
	kg.		*kg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
120	318	1: 7.0	—	28	16.4	3.68	2.5	1.1	2.3	Jersey.
122	560	1: 4.7	1.61	27	10.5	4.64	1.9	1.2	2.2	Holstein. Calf 4 months old.
121	545	1: 4.7	1.61	25	13.1	3.34	1.9	1.3	2.4	"
123	560	1: 8.1	1.00	25	9.8	3.46	2.6	1.1	2.2	"
127	—	1: 7.6	0.96	25	10.5	3.30	1.7	1.2	2.2	" Calf 4 months old.
124	550	1: 8.1	1.00	19	8.2	3.45	1.8	1.2	2.1	" " 7 " "
126	—	1: 10.9	0.66	18	6.8	2.6	1.6	1.2	2.0	" " 7 " "

In Table IV are shown results obtained on a single cow whose milk we were able to examine at more or less regular intervals for a period of several weeks after delivery. This experiment was carried on during the winter months (February and March) and the analyses were started within 3 or 4 hours after the milk had been drawn. It was not possible to arrange to have this animal fed on a weighed ration, so that we are not able to make any definite statement on this point, except to say that with the exception of the first few days after delivery the food remained the same during the remainder of the time that we were making examinations of the milk, and that it was rather high in protein. The validity of the extremely high values for non-protein nitrogen and amino nitrogen noted during the first few days after calving might well be questioned as due to incomplete precipitation of the proteins of colostrum by the methods employed. It should be pointed out, however, that the values for urea nitrogen (which is determined on the entire milk without the necessity of precipitating the proteins) are also extremely high. In the case of another cow from which we were able to obtain samples of milk at more or less regular intervals for a period of 4 days after delivery results were obtained substantially similar to those given in Table IV.

The data collected on this point are difficult to explain. A temporary impairment of renal function during the later stages

of pregnancy, the passage into the blood stream of nitrogenous material formed during the involution of the uterus, or perhaps a combination of both factors might be involved. Studies on human milk, the results of which will be published shortly, lead us to believe that the concentration of many of the non-protein nitrogenous constituents of milk are on the same level as in the maternal blood; it would, therefore, appear to be of considerable interest to repeat the above observations, and at the same time make a series of analyses of the blood. As the experimental material is not available we have been unable to follow out the suggestion.

TABLE IV.

Changes in Content of the Non-Protein Constituents of Cow's Milk during the First Four Weeks after Calving.

Time after calving	Amount per 100 cc. of milk.					
	Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.	Preformed creatinine.	Creatine.
hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
2	74	28.6	7.8	1.5	—	—
14	60	28.0	8.0	1.7	1.3	2.7
26	56	28.0	7.7	1.8	1.3	2.5
36	48	27.0	7.7	1.8	1.2	2.4
50	48	27.0	7.7	1.6	1.2	2.3
74	48	26.6	7.1	1.8	1.2	2.0
98	48	26.0	5.1	1.7	1.3	2.0
122	44	20.0	5.4	1.8	1.3	2.0
146	44	20.6	5.1	1.6	1.2	2.0
432	34	16.6	4.4	1.8	1.3	2.0
668	34	16.0	3.6	1.6	1.3	2.0

SUMMARY.

Figures are given for the non-protein nitrogen, amino nitrogen, urea, uric acid, creatinine, and creatine in cow's milk. It has been shown that the content of non-protein nitrogen, amino nitrogen, and urea is influenced by the character of the food, being increased in high protein feeding and decreased when rations of low protein content are given. The non-protein nitrogen, amino nitrogen, and urea content of colostrum is high and approaches normal values only on the fourth day after delivery.

A REVISED COLORIMETRIC METHOD FOR DETERMINATION OF URIC ACID IN URINE.

By OTTO FOLIN and HSIEN WU.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, May 31, 1919.)

For several years acid silver lactate has been used in this laboratory, instead of ammoniacal silver solutions, for the precipitation of uric acid, but publication of the method has never seemed worth while so long as the most cumbersome feature of the uric acid determination, namely the preparation of a permanent standard uric acid solution, had not been solved. A standard uric acid solution of practically unlimited keeping quality was recently described by us[1] in connection with the determination of uric acid in blood. The adaptation of the colorimetric method for uric acid described in this paper has been tested out by ourselves and has also proved thoroughly satisfactory in the hands of our medical students.

Solutions Required.

1. Standard uric acid solution. Before starting to prepare the uric solution a 20 per cent filtered solution of sodium sulfite should be available. Dissolve 1 gm. of uric acid in 125 to 150 cc. of 0.4 per cent lithium carbonate solution and dilute to a volume of 500 cc. Transfer 50 cc. corresponding to 100 mg. of uric acid to each of a series of volumetric liter flasks. Add about 300 cc. of water and then add 500 cc. of clear 20 per cent sodium sulfite solution, mix, dilute to volume, and mix thoroughly. Fill a series of 200 cc. bottles, and stopper very tightly. The reason why a series of small bottles is used as containers is, of course, to reduce the absorption of oxygen from the air.

2. A 10 per cent sodium sulfite solution, kept like the uric acid solution, in small tightly stoppered bottles.

[1] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 102.

3. A 5 per cent sodium cyanide solution.

4. A solution containing 5 per cent of silver lactate and 5 per cent of lactic acid.

5. The uric acid reagent of Folin and Denis. Boil 100 gm. of sodium tungstate with 80 cc. of phosphoric acid (85 per cent) and 700 cc. of water for not less than 2 hours and dilute to 1 liter.

Transfer from 1 to 3 cc. of urine to a 15 cc. centrifuge tube and mix with enough water to make a volume of about 6 cc. Add 5 cc. of the acid silver lactate solution and stir with a very fine glass rod (diameter 1 to 2 mm.). Rinse off the rod with a few drops of water and centrifuge. If enough silver solution has been added the precipitate settles very quickly. Add a drop of silver lactate solution so as to be sure that an excess is present; if a precipitate is formed add more (2 cc.) of the silver lactate solution and centrifuge again. In point of fact the first 5 cc. addition of silver lactate is almost always enough, but it is not safe to omit the test. Pour off the clear supernatant liquid as completely as possible.

To the precipitate in the centrifuge tube add, from a burette, 4 cc. of 5 per cent sodium cyanide solution and stir until a perfectly clear solution is obtained. Pour the contents into a 100 cc. volumetric flask and rinse tube and stirring rod, using for this purpose about 15 to 25 cc. of water. Add 5 cc. of 10 per cent sodium sulfite solution (to balance the sulfite in the standard uric acid solution). Dilute to a volume of about 50 cc.

Transfer to another 100 cc. flask 5 cc. of the standard uric acid sulfite solution, containing 0.5 mg. of uric acid; add 4 cc. of the cyanide solution and dilute to about 50 cc. Then add 20 cc. of saturated sodium carbonate solution to each flask, mix, and finally add *with shaking* 2 cc. of the uric acid reagent. Let stand for 3 to 5 minutes, fill to the mark, mix, and make the color comparison in the usual manner, never omitting to first read the standard against itself. Artificial light (with "daylite" glass) is better than day light for this color comparison.

With the standard set at 20 mm., 10 divided by the reading of the unknown (in mm.) gives the amount of uric acid (in mg.) in the volume of urine taken.

The discarded blue solution should be poured as directly as possible into the drain pipes of sinks on account of the cyanide.

A SIMPLIFIED MACRO-KJELDAHL METHOD FOR URINE.

By OTTO FOLIN and L. E. WRIGHT.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, May 31, 1919.)

The purpose of this communication is to describe a macro-Kjeldahl method for the determination of nitrogen in urine which requires very little equipment and by which a urinary nitrogen determination can easily be finished in 20 to 25 minutes.

The hydrolyzing-oxidizing reagent is the same phosphoric-sulfuric acid mixture that is used in the micro method. To 50 cc. of 5 to 6 per cent copper sulfate solution add 300 cc. of 85 per cent phosphoric acid and 100 cc. of concentrated sulfuric acid. 5 cc. of this mixture are used for the destructive digestion of 5 cc. of undiluted urine. 10 per cent solution of ferric chloride is also required. The ferric chloride can scarcely be considered indispensable; but it hastens the digestion, and the iron hydroxide promotes even boiling during the subsequent distillation.

Transfer 5 cc. of undiluted urine to a 300 cc. Kjeldahl flask (Pyrex). Add 5 cc. of the phosphoric-sulfuric acid mixture, also 2 cc. of 10 per cent ferric chloride solution and 4 to 6 small pebbles (to prevent bumping). Boil in a hood over a microburner.[1]

Microburners are by far the best to use as a source of heat, because even inexperienced workers or students can not go far astray with them; whereas, large Bunsen burners with their great variations in heating power are much more difficult to regulate. The microburner should give a good strong flame and the top of the burner should be not more than 1 cm. away from the bottom of the flask.

Boil vigorously. In 3 to 4 minutes the foam which forms at first will entirely disappear and the flask becomes filled with dense

[1] A very convenient clamp for holding the Kjeldahl flasks in position is the one listed as No. 24598 in Arthur H. Thomas Company's catalogue. The microburner which we use is listed under No. 1506 in Eimer and Amend's catalogue.

white fumes. When this stage is reached (but no earlier) cover the mouth of the flask with a small watch-glass and continue the vigorous heating for 2 minutes. At the end of 2 minutes dilute urines will already be green or blue and concentrated urines will be a light straw yellow, the black carbonaceous matter being completely destroyed. The flame should then be turned very low and the gentle boiling process should be continued for 2 minutes, making a total boiling period of 4 minutes, counting from the time the watch-glass was put in place. Remove the flame and let the flask cool for 4 to 5 minutes. At the end of 4, or not more than 5 minutes, add first 50 cc. of water, then 15 cc. of saturated sodium hydroxide (50 to 55 per cent), and connect the Kjeldahl flask promptly by means of a rubber stopper and ordinary glass tubing with a receiver containing from 35 to 75 cc. of 0.1 N acid together with water enough to make a total volume of 150 cc., and a drop or two of alizarin red. Florence flasks, capacity 300 cc., of Pyrex glass make excellent receivers for this distillation. As soon as the connection is made with the receiver apply the flame again at full force, but not directly under the center until the acid and alkali in the flask have had time to mix. The contents in the flask begin to boil almost at once and 4 to 5 minutes boiling transfers the whole of the ammonia to the receiver. The contents in the receiver become heated of course, since no condenser is used, but under the conditions described the temperature reached is only 65–70°C.

The only precaution needed in connection with the titration of the distillate (without previous cooling) is that a faint red color shall be accepted as the end-point. The color will deepen on cooling, and when time permits it is more satisfactory to cool in running water before titrating.

While the operator is directed to make prompt connections with the receiver after the alkali has been added to the digestion mixture there is in point of fact very little danger of losing ammonia vapors by being unduly slow in closing the mouth of the flask. Similarly when the receiver contains too little acid and turns pink there is no need for extraordinary haste in adding more acid. The water, though warm, will hold considerable free ammonia.

It will be noted that the digestion period, 30 to 40 minutes of ordinary Kjeldahl determinations, has here been cut down to 4

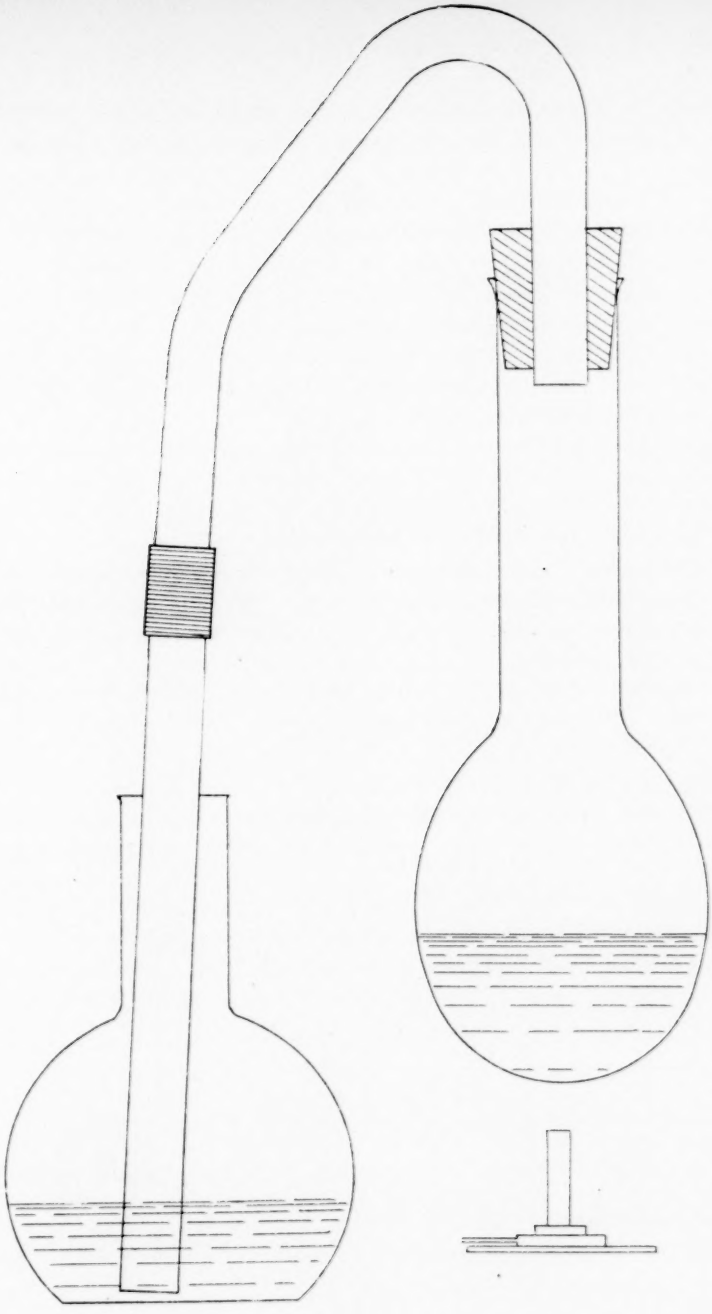

FIG. 1.

minutes and that the distillation period, 25 to 40 minutes, has been cut down to 5, and the only source of heat is an ordinary microburner. The delivery tubes are made from glass tubing, small enough to pass into the ready-made holes in rubber stoppers. For the sake of flexibility the delivery tube must consist of two parts connected with a short piece of rubber tubing (see Fig. 1). It may also be observed that the Kjeldahl flask need not be moved from the time the digestion is begun until the determination is finished, though this involves the continuous use of the hood. Where hood space is scarce the distillation can, of course, be conducted just as well at the desk.

One very satisfactory feature of this new process is that the preliminary steaming out of the condenser for an occasional nitrogen determination is replaced by a simple rinsing of the glass tube.

The simplified distillation process described above can also be used in other determinations involving the removal of ammonia; as, for example, in urea determinations. The elaborate metal condensers now found in every well equipped laboratory are practically superfluous.

The method described in this paper, as far as the destructive digestion is concerned, is primarily intended for urine only. It is not applicable to highly resistant materials, as for example milk, which cannot be completely destroyed within 6 minutes. Urines containing much sugar belong in this class. If fuming sulfuric acid be substituted for ordinary sulfuric acid in the preparation of the hydrolyzing reagent, or if 2 cc. of fuming sulfuric acid are used in addition to 5 cc. of the regular reagent, sugar urines are readily destroyed within the required heating period of 4 to 5 minutes.

THE VITAMINE REQUIREMENT OF YEAST.

A SIMPLE BIOLOGICAL TEST FOR VITAMINE.*

By ROGER J. WILLIAMS.

(From the Hull Laboratories of Physiological Chemistry and Pharmacology, University of Chicago, Chicago.)

PLATE 6.

(Received for publication, May 2, 1919.)

INTRODUCTION.

Evidence is here presented, based upon direct microscopic observation of the growth of individual yeast cells, that the water-soluble-, beri-beri-preventing vitamine, relatively so abundant in yeast, is necessary for the nutrition of yeast cells themselves.

Pasteur in his famous researches found that yeast would grow in a solution of cane sugar, ammonium salt, and the salts of yeast ash. Since his day it has been generally believed that yeast can grow on such a synthetic medium and that an addition of broken down protein improves such a culture medium for yeast. Pasteur, however, observed the fact that a particle of yeast the size of a pin head (containing several million cells) must be used to inoculate such a solution in order to get appreciable growth and fermentation. This observation we shall see is a very important one.

Wildiers[1] made this same observation in 1901. He also observed that, by adding a little sterile water extract of yeast to a medium containing ammonium salt as its only source of nitrogen, a very small amount of yeast could be made to grow rapidly as judged by carbon dioxide production. This fact he thought to be due to a substance in the yeast extract which in addition to other known nutrients had to be supplied for the nutrition of the yeast cells. This new substance indispensable for the growth of yeast Wildiers called "bios." He found the substance to be soluble in water and 80 per cent alcohol but insoluble in ether. It was dialyzable and it was not precipitated by any of the ordinary precipitants including

* This work was carried out in connection with a fellowship for the study of Yeast Nutrition given by the Fleischmann Company.

[1] Wildiers, E., *La Cellule*, 1901, xviii, 313.

phosphotungstic acid. The substance was stable in acid solution but was destroyed by short boiling in dilute alkali. This instability was not uniform as he obtained very divergent results. Wildiers found "bios" to be absent from acid-hydrolyzed egg albumin, and from yeast ash.

Devloo[2] in 1906 claimed to have prepared "bios" in a state of purity from lecithin. Judging from his own statements, however, the material he prepared was in reality not very active in promoting yeast growth. His work will be referred to later in the discussion.

Pringsheim[3] in 1906 showed that in the synthetic solution referred to by Wildiers there was unquestionably some growth of single yeast cells, though the growth was small. He sought to refute the work of Wildiers by saying that "bios" was nothing more nor less than protein material which theoretically should be obtained in most available form for yeast, from yeast itself. Evidently this argument stood, as in more recent years no mention of Wildiers' work has been found.

In this laboratory a study of the nutrition of yeast was undertaken with the intention of first studying solutions of known composition. In working with synthetic solutions it was also thought desirable to first obtain a pure culture of yeast grown on a synthetic medium if such a thing were possible. However, using the Lindner droplet method the single cell from which the pure culture was to be grown in such a medium could never be made to produce a colony large enough to be seen with the naked eye, and consequently such a culture was out of the question. Bearing in mind Wildiers' work, other experiments along the same line were performed with the result that Wildiers' observations were largely confirmed. The possibility presented itself that the substance which Wildiers called "bios" might be the same as the vitamine known to be contained in yeast. With this possibility in mind, a preparation of "activated" fullers' earth was obtained through the courtesy of Williams & Seidell and it was found to contain a substance which in small amounts would promote remarkably the growth of single yeast cells. The other work reported below is the logical outgrowth of this initial observation.

EXPERIMENTAL.

I. General procedure.—In preliminary experiments fairly uniform results were obtained by using merely a suspension of commercial pressed yeast for seeding, but for all the experiments

[2] Devloo, R., *La Cellule*, 1906, xxiii, 361.

[3] Pringsheim, H. H., *Centr. Bakteriol., 2te Abt.*, 1906, xvi, 111.

here reported a pure culture of baker's yeast was used. All experiments except under VI were conducted with a pure culture grown in this laboratory. In these experiments a pure culture was used which was kindly furnished by Dr. R. E. Lee of the Fleischmann Company. The results obtained on this culture in that study were also confirmed with the other culture.

In order to maintain a pure culture during the necessary manipulation most of the work was carried on inside a sterile cupboard made for the purpose. This glass cupboard was sprayed inside with dilute alcohol and closed generally a day or more previous to its use. The sliding glass door was opened during the work only enough to allow free movements of the hands of the experimenter.

The greatest difficulty in carrying out the experiments reported below, and in obtaining uniform results, lay in obtaining yeast in the right condition so that the colonies would break apart easily when a suspension was made and at the same time contain a large proportion of live cells. If, for instance, yeast which had grown one day was suspended, the yeast cells stayed in colonies and if a suspension was shaken hard enough to break the cells apart most of the cells were killed or their vitality was so lowered that they did not grow under the conditions of the experiment. In the case of yeast which has grown for a week or more at 30°C. or a shorter time at 37°C. the cells of colonies break apart into single cells easily, but due to autolysis, a considerable number of the cells are dead, and those which do grow, grow distinctly more in a solution containing ammonium salt as the only source of nitrogen than do cells when autolyzed material is not present. The autolyzed material of such a culture contained in a speck of yeast much smaller than a pin head when added to 25 cc. of solution can be detected by the increased growth of cells in the hanging drops. For the experiments reported the yeast used was grown 3 or 4 days at 30°C. and in most cases kept in the refrigerator a day or two before being used. In such a growth the cells often had some tendency to remain in clusters but it was much more easily worked with than younger yeast. The yeast at best seemed to vary somewhat in its properties when grown apparently under the same conditions. At times during the investigation the yeast was easier to work with than at other times. All the

yeast used in the experiments reported was grown on a rich malt wort obtained from a Fleischmann factory, and solidified with agar. When yeast was grown on a wort which had been autoclaved a long time or many times the growth of cells in a solution of mineral salts and sugar was still less than that reported in the case of the more vigorous yeast.

In the preliminary experiments various solutions were used, in which asparagine and ammonium lactate were used as sources of nitrogen, but in the experiments which are reported here the simpler salt $(NH_4)_2SO_4$ was used. The solution used consisted of the following substances dissolved in a liter of distilled water:

20	gm. cane sugar.
3	gm. $(NH_4)_2SO_4$
2	gm. KH_2PO_4
0.25	gm. $CaCl_2$
0.25	gm. $MgSO_4$

Yeast cells are not sensitive to small changes in hydrogen ion concentration; nevertheless, the phosphate buffer with the very small additions made served to control the acidity of the medium.

The solutions to be tested were always sterilized, ordinarily at 10 pounds pressure for 10 minutes before being seeded with yeast. In some cases the solutions were kept in the refrigerator 2 or 3 days after sterilization before the experiment was carried on. Bacterial contamination was carefully avoided in all material. When in preliminary experiments a "mineral salt solution" became contaminated yeast cells grew in the solution better than before contamination, although the solution was sterilized before being seeded with yeast. Hence it was necessary to avoid foreign organisms and the nature of the method made it possible to be sure that this factor was controlled.

When the yeast culture and the solution were in proper condition the seeding was carried out in the sterile cupboard mentioned above. A small amount of yeast was taken out of the culture and suspended in a test-tube containing about 10 cc. of sterile water. The amount of yeast put in approximated the size of a pin head, but could be estimated only after a little practice. The suspension was then slightly cloudy. A 1 cc. sterile pipette was introduced into the suspension and attached

to a rubber tube with a pinch-cock. The rubber tube contained a good cotton plug. By blowing through the tube and pipette a uniform suspension of the yeast was insured at the moment the pipette was filled. 1 cc. of the suspension was then put for trial into a flask containing the same amount of water as the solutions to be tested. After gently shaking the very dilute suspension a sterile steel pen was dipped into it and 25 drops were made on a cover-slip and immediately inverted on a hollow ground slide prepared with vaseline for sealing the chamber air-tight.[4] The drops were then examined under the low power of the microscope to see if the seeding was about the right amount. If the seeding was much too great it was corrected by diluting the suspension with sterile water and if too small by using more of the suspension for seeding or adding more yeast to the suspension. Ordinarily there was no difficulty in obtaining the right amount of seeding as it is not necessary to have an exact and definite concentration of cells. The number of cells in the hanging drops can be varied by varying the time interval between shaking the solution and dipping the pen. If slightly too many cells are present in the drops when the pen is dipped immediately after shaking, waiting a moment before dipping the pen will give the desired results. When the correct seeding was found, each solution to be tested was seeded immediately in the same way. In this way the yeast to be seeded remained in the distilled water only a few minutes.

At least 50 hanging drops on two slides were made in the way described from each solution to be tested. By using a standard pen the drops were of fairly uniform size and by having the solution as cool as possible evaporation was avoided. The contents of each drop which contained one single cell, two cells joined together, or one single and one double cell or two of each and no more, were recorded on squared paper. In order to increase the number of observations a single cell with a small bud was counted as a single cell and a double cell with a bud was counted as a double cell. This did not change the trend of the results as all solutions were treated alike in this manner and no conclusions are drawn from differences unless they are large.

[4] Thanks are due Dr. Lee for his suggestion of the hanging droplet method of study.

The individual variations of the yeast cells are quite large as will be seen in the results below. Many observations showed that yeast cells grow practically as well whether there are one or two cells in a drop provided the growth is not carried too far. After the contents of each slide had been recorded the slide was put into an incubator at 30°C. and the time of incubation recorded. In this manner eight satisfactory slides of 25 drops each representing four solutions could be made, and the contents recorded in an hour.

After the cells had been growing 5 or 6 hours all the slides were examined. Slight growth had taken place in this time and the records of the contents of the drops were verified and the growth was recorded. In case any cells were overlooked they were noted and if more than two colonies were located in one drop, the particular drop was excluded from consideration. At the end of 20 to 24 hours the drops were examined and counts again made. If a colony could not be counted because of the presence of some debris (for instance flakes from the steel pen) it was excluded from consideration. This was also true if it could not be determined with a good degree of certainty whether a certain colony was produced by a single or double cell or both, both of which might have been in the same drop. Ordinarily, however, few drops were excluded and practically all the original cells were considered. No colonies were excluded because of irregularities of growth. In case the colonies were small the count could be accurate; larger colonies were less accurately determined as the colonies grow to some extent in three dimensions. Estimations of the larger colonies were made by counting a portion of the colony and estimating the number of times it was contained in the whole. An effort was made to make the estimates on large colonies conservative.

Often the contents of the drops were watched for several days but the results are not reported unless they have some added significance. In some experiments there were practically no single cells present, and in others few double cells were present, consequently only one kind is reported in the experiments, provided there were sufficient numbers to warrant drawing conclusions. The growth of single and double cells was always parallel.

II. Protein-Free Milk.—Osborne and Mendel have used protein-free milk extensively as a source of the water-soluble vitamine.

This experiment was performed to find out if the substance promoting the growth of yeast is also present in such material. 200 cc. of milk were made "protein-free" following the method of Osborne and Mendel.[5] The filtrate which was water-clear was evaporated to dryness on a water bath in a current of air. It was further dried by alcohol and ether treatment and kept in a vacuum desiccator. This material was extracted three times with warm ether and three times with a like quantity of warm 95 per cent alcohol. The ether extract which was very slight in amount was digested with water and made up to the original volume of

TABLE I.

	Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 1 cc. H_2O	20	12	15	4	8
II.	25 cc. control solution + 1 cc. ether extract of protein-free milk	14	4	8	4	6
III.	25 cc. control solution + 1 cc. alcohol extract of protein-free milk	18	10	180	36	108

the milk. The alcoholic extract which was much larger in amount was also digested with water and made up to the original volume of the milk.

The solutions to be tested were made up as indicated in Table I, sterilized, and seeding carried out in the manner described. Growth at the end of 22 hours was recorded. The results show plainly that the growth stimulant is present in the alcohol fraction of protein-free milk.

III. Wheat Germ.—Wheat germ has been used as a source of the water-soluble vitamine by McCollum and his coworkers as well as others.

[5] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, pts. i, ii, 1911.

In an experiment not performed specifically for this purpose and only part of which is reported (Table II), it is shown that wheat germ may be used as a source of the substance promoting the growth of yeast.

Wheat germ was extracted three times with a good quantity of hot 95 per cent alcohol, the extract was filtered, and the filtrate evaporated to dryness. The residue was digested as completely as possible in such a quantity of water that 1 cc. of the solution was equivalent to 75 mg. of the original wheat germ. After filtration 2 cc. of the solution were used for the experiment.

The results recorded in Table II were obtained at the end of 22 hours growth. There is evidence of a considerable amount of the yeast growth substance present in wheat germ.

TABLE II.

	Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 2 cc. H_2O	20	2	3	2	2.5
II.	25 cc. control solution + 2 cc. alcoholic extract of wheat germ.	18	8	135	54	91

IV. Lactose.—McCollum and Davis[6] found in feeding experiments, in which they used 20 per cent of Kahlbaum and Merck's lactose, that the lactose contained the water-soluble vitamine as an impurity. We ran tests to ascertain if the yeast growth substance was likewise present in lactose as an impurity.

10 gm. of Kahlbaum's lactose were extracted with 95 per cent alcohol continuously for 6 hours. The alcohol was cooled, filtered free from lactose crystals, and evaporated to dryness on the water bath. The residue was then dissolved in 10 cc. of water. 5 cc. of this solution were used for each of the experiments reported below. The two experiments are used together as neither one by itself was absolutely conclusive, due to the low vitality of the yeast used. The two experiments were conducted in the same way except that the yeast used in the second case had been in the refrigerator one day longer and 2 cc. of a suspension instead of 1 cc. were used for seeding.

[6] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 183.

The results of growth at the end of 22 hours of both double and single cells are reported in Table III and show definitely the presence of a small amount of the growth-promoting substance in Kahlbaum's lactose.

V. Pancreas Tissue.—Eddy[7] has found the water-soluble vitamine to be present in fairly large quantities in pancreas tissue.

This experiment was performed to determine whether the yeast growth substance is also present in pancreas tissue, pancreatin U. S. P. being used as a convenient source. 2 gm. of pancreatin were extracted directly with warm ether and filtered through a good filter. The residue was then extracted in like

TABLE III.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 5 cc. H_2O	22	3	12	7	9
II. 25 cc. control solution + 5 cc. alcoholic extract of lactose	29	12	50	18	36
	Single cells considered.				
I. 25 cc. control solution + 5 cc. H_2O	8	4	10	3	6
II. 25 cc. control solution + 5 cc. alcoholic extract of lactose	9	3	36	10	23

manner with warm 95 per cent alcohol and filtered. Each filtrate was evaporated to dryness and dissolved in 20 cc. of water. 1 cc. was used in the experiments which was equivalent to 0.1 gm. of pancreatin.

Table IV gives the results of a 22 hours growth following the usual procedure and shows the presence of a considerable amount of the yeast growth substance.

VI. Hydrolyzed Casein.—The purpose of this experiment was to test the possibility that the growth-promoting substance of yeast might be some of the amino-acids derived from an "adequate" protein like casein.

[7] Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.

5 gm. of casein Merck (Hammarsten) were digested with 25 per cent H_2SO_4 for 14 hours using a reflex condenser. The bulk of the acid was removed with $Ba(OH)_2$ and the solution brought to very slight acidity. This was made up to 500 cc. volume and sterilized on two occasions at 10 pounds pressure for 10 minutes. When ready for use, to a portion was added crystalline tryptophane up to 1.5 per cent of the original casein digested. The tryptophane had been prepared from casein by the Hopkins method. It is obvious from the previous experiment why pancreatin was not used to digest the casein. For purposes of comparison solutions were used which were known to contain the growth-promoting substance, an alcoholic extract of an alkali extract of "activated" fullers' earth being used as a source of the

TABLE IV.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth	Average growth.
I. 25 cc. control solution + 1 cc. H_2O	14	8	10	4	5.6
II. 25 cc. control solution + 1 cc. ether extract of pancreatin	13	6	15	5	9.2
III. 25 cc. control solution + 1 cc. alcoholic extract of pancreatin	15	13	280	80	187

growth-promoting substance. Each cc. added contained 1 mg. of actual material. Because a considerable amount of the casein digest was added in this experiment additional phosphate buffer was used to control the acidity.

Table V gives the results for the first 5 hours growth and Table VI for the first 22 hours.

Noting the fact that the growth in Solution IV had slowed up it was thought desirable to watch it another day.

At the end of 48 hours the growth of the three colonies in each solution which had shown maximum growth in 22 hours were counted or estimated (Table VII).

In Solution IV the initial growth was most rapid as would be expected, but later was markedly retarded. The results clearly indicate that as the yeast grows in the solution containing both

TABLE V.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth	Average growth.
I. 25 cc. control solution + 1 cc. 10 per cent KH_2PO_4 solution + 11 cc. H_2O	17	2	3	3	3
II. 25 cc. control solution + 1 cc. 10 per cent KH_2PO_4 solution + 1 cc. fullers' earth extract	16	11	6	3	4.0
III. 25 cc. control solution + 10 cc. casein digest + tryptophane + 1 cc. H_2O	13	9	5	3	3.6
IV. 25 cc. control solution + 10 cc. casein digest + tryptophane + 1 cc. fullers' earth extract	15	12	10	3	6.1

TABLE VI.

Solution	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I	17	6	9	4	5.8
II	16	15	95	12	58.0
III	13	12	10	3	8.9
IV	15	14	46	17	32.0

TABLE VII.

Solution.	Average of the three maxima.	
	22 hours.	48 hours.
I	8	11
II	75	283
III	10	13
IV	43	45

casein digest and the growth-promoting substance, something harmful is formed which is not initially present. This may throw some light on the observation of Effront as well as others that too complete hydrolysis of protein injures it as a yeast food.

Aside from this question the experiment shows that the growth-promoting substance is not one of the commoner amino-acids known to be contained in an acid digest of casein.

VII. Adsorption from Malt Wort by Fullers' Earth.—Seidell[8] has found that the water-soluble vitamine is adsorbed nearly quantitatively from autolyzed yeast filtrate by certain varieties of fullers' earth and is thus removed from the bulk of the original material.

As has been mentioned it has been found that such a preparation contains the substance which stimulates yeast growth in relatively large quantity. The experiment reported below was performed to confirm this fact and to determine if there is anything unadsorbed by the fullers' earth which may take the place of the adsorbed material.

Fresh malt wort obtained from one of the Fleischmann factories was evaporated on the steam bath to about one-half its original volume. It was filtered and a portion diluted 1:20, while a 25 cc. portion was shaken continuously with 1 gm. of fullers' earth (Eimer and Amend) for 1 hour. The fullers' earth was filtered off, the filtrate diluted 1:20, and the fullers' earth washed once with water. The fullers' earth was then extracted by shaking 10 minutes with saturated $Ba(OH)_2$, filtered, and the barium removed with H_2SO_4. The resulting solution was diluted so that 2 cc. were equivalent to 3 cc. of the diluted wort. This experiment was carried on with the pure culture furnished by the Fleischmann Company using the general method previously described.

Table VIII gives the results of 6 hours of growth.

At the end of 24 hours, due to the large number and size of the colonies, only the three in each solution which had shown maximum growth in 6 hours were considered (Table IX).

One effect is observed here but not recorded in any other experiments. The yeast used for seeding had remained in the

[8] Seidell, A., *Bull. Hyg. Lab., U. S. P. H.*, 1916, xxxi, 364.

refrigerator a week, during which time autolysis had evidently taken place. A large percentage of cells was not able to grow even in the good medium. Furthermore, the growth of those which did grow in the control solution was greater than in other

TABLE VIII.

Composition of solution.	Single cells considered.	Number growing.	Maximum growth	Minimum growth	Average growth.
I. 25 cc. control solution + 5 cc. H_2O	25	10	4	2	2.4
II. 25 cc. control solution + 3 cc. diluted fullers' earth-treated wort + 2 cc. H_2O	22	10	8	2	3.2
III. 25 cc. control solution + 3 cc. diluted fullers' earth-treated wort + 2 cc. alkaline extract of fullers' earth	19	11	10	2	5.5
IV. 25 cc. control solution + 3 cc. original untreated diluted wort + 2 cc. H_2O	23	15	13	2	5.6

TABLE IX.

Solution.	Number considered.	Number growing.	Growth of the three maxima.			Average growth of the three maxima.
I	25	13	2	16	10	15
II	22	11	51	40	42	44
III	19	12	180	300	180	220
IV	23	12	200	375	300	292

experiments, due evidently to the autolyzed material added with the live cells. That the effect is not due to the vigor of the yeast was shown by the fact that the same yeast was used in a previous unreported experiment, where, before the slight autolysis had taken place, the cells had grown much less than in this experiment.

The foregoing experiment shows that the growth-promoting substance is taken out by shaking a solution with fullers' earth, just as vitamine is known to be. Probably it can be removed even more quantitatively by observing the right conditions of acidity, quantity of earth used and time of shaking; conditions which as yet have not been definitely determined.

VIII. Phosphotungstic Acid Precipitation.—Since the earliest work of Funk, precipitation by phosphotungstic acid has been used as a means of purification of the vitamine which prevents beri-beri. Wildiers found the so called "bios" not to be precipitated by phosphotungstic acid. The conditions of precipitation which he used were not definitely stated. Consequently, the experiment was repeated to determine if under proper conditions the yeast growth substance can be precipitated with phosphotungstic acid.

20 gm. of "activated" fullers' earth were shaken with 200 cc. of saturated $Ba(OH)_2$ for 5 minutes and filtered. The filtrate was brought with H_2SO_4 to very slight acidity, filtered, and the filtrate concentrated *in vacuo* to about 40 cc. and filtered from organic residues. The filtrate was divided into two equal portions. To one portion was added 50 per cent H_2SO_4 until it contained 5 per cent acid, and to it was added as a precipitant altogether 10 cc. of a 20 per cent solution of phosphotungstic acid dissolved in 5 per cent H_2SO_4. This was a slight excess of phosphotungstic acid. It was allowed to stand over night. The precipitate of phosphotungstates was filtered off and washed thoroughly with 5 per cent H_2SO_4 containing 0.5 per cent of phosphotungstic acid. The filtrate was made slightly alkaline with $Ba(OH)_2$ and filtered, the precipitate being washed thoroughly with half saturated $Ba(OH)_2$. The filtrate was then made very slightly acid, filtered, and after dilution to 200 cc. was used in the experiment as the "phosphotungstic acid filtrate." The phosphotungstates were decomposed with five 20 cc. portions of saturated $Ba(OH)_2$ grinding each time in a mortar. The material was in contact with the alkali a total of over an hour and the last two portions of alkali were warmed gently with the precipitate. The material was filtered and the precipitate washed on the filter with saturated $Ba(OH)_2$. The filtrate was made very slightly acid with H_2SO_4 and again filtered.

This was also diluted to 200 cc. and used in the experiment as "decomposed phosphotungstates." The original untreated solution was likewise diluted and equivalent amounts (2 cc.) of each solution were used. Each portion added was equivalent to 0.1 gm. of the "activated" fullers' earth. Each of the solutions had been heated in the autoclave a total of 10 minutes at 10 pounds pressure.

Hanging drops were made in the usual way. Table X shows the results at the end of 24 hours.

To get the best quantitative interpretation of the results the number growing in the solution should be considered as well as

TABLE X.

Composition of solution.	Single cells considered.	Number growing.	Maximum growth	Minimum growth	Average growth.
I. 25 cc. control solution + 2 cc. H_2O	16	3	5	2	3.3
II. 25 cc. control solution + 2 cc. original untreated extract	15	13	70	14	35.
III. 25 cc. control solution + 2 cc. "phosphotungstic acid filtrate"	18	4	33	2	25.
IV. 25 cc. control solution + 2 cc. "decomposed phosphotungstates"	14	10	70	11	51.

the average growth. Solution IV was most active, Solution II next in activity, and III much weaker as shown by the small percentage which grew. The "decomposed phosphotungstates" show greater activity than the original extract. Later the explanation for this result was found in the effect of acid and alkali on the material and will be discussed later. The activity of all the solutions except Solution I was considerably less than would be expected from the amount of earth used, judging by other experiments. Possibly some activity was lost when the original material was filtered after concentration *in vacuo*. That the substance is precipitated by phosphotungstic acid cannot be doubted. A similar result to that reported was obtained in other

experiments, the precipitation being more incomplete with more dilute solutions.

IX. Heat Stability.—Considerable divergence of opinion exists as to the degree of heat stability of the water-soluble vitamine. The more recent work indicates that in foods and in ordinary preparations it is quite stable to heat, not being destroyed at boiling temperature and withstanding considerable heating under pressure. No doubt preparations vary depending on various factors.

An alcoholic extract of dried yeast was tested for the heat stability of the substance promoting the growth of yeast. To each of two flasks were added 6 cc. of a solution of yeast extract, which had been prepared by extracting dry yeast with 95 per cent

TABLE XI.

	Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 6 cc. H_2O	12	3	4	3	3.3
II.	25 cc. control solution + 6 cc. autoclaved extract	20	12	6	3	4.3
III.	25 cc. control solution + 6 cc. untreated extract	11	7	10	3	5.6

alcohol, and to a third flask 6 cc. of distilled water. Each of the samples of yeast extract was equivalent to 0.3 gm. of dry yeast. One of the portions of extract was heated in the autoclave for 30 minutes at 15 pounds pressure and brought to its original weight with distilled water. To each of the three flasks were then added 25 cc. of the control solution and they were sterilized as usual for 10 minutes at 10 pounds pressure. After 6 hours growth in hanging drops results were obtained as recorded in Table XI.

At the end of 23 hours estimations were made on the three colonies in each solution which in 6 hours had shown the maximum growth (Table XII).

The substance promoting the growth of yeast in the condition in which it was used was partially destroyed by heating under 15 pounds pressure for 30 minutes.

X. Treatment with Acid or Alkali.—The water-soluble vitamine has been found to be quite stable in acid solution even at the boiling temperature. Vedder and Williams[9] found it to be markedly changed in its physiological properties by acid treatment and suggested a hydrolysis as a possible explanation. On the other hand it has been found to be unstable in alkaline solution. On this point there has been a wide divergence of results. At least one worker has reported its destruction by as weak a base as ammonia. McCollum and Simmonds[10] found that as prepared from wheat germ it was destroyed by short boiling with very dilute alkali. Williams and Seidell[11] have found the preparation extracted from "activated" fullers' earth to be quite stable to alkali, though the alkali alters it in some way without destroying its property of preventing polyneuritis in pigeons. Again a hydrolysis was suggested to explain the change. Recent

TABLE XII.

Solution.	Growth of three maxima.			Average of three maxima.
I	9	8	10	9
II	160	180	150	163
III	400	320	200	307

work by Daniels and McClurg[12] has shown that as the vitamine exists in foods it is not readily destroyed by cooking under pressure with very dilute alkali.

The study of the behavior of the growth-promoting substance of yeast toward acid and alkali has proven to be a complicated problem, due apparently to the interference of toxic substances formed. This study is therefore not complete although a large number of experiments has been performed. An indication of the trend of the results will be given.

The fullers' earth preparation has been found to be quite stable in alkaline solution, confirming the observations of Williams and

[9] Vedder, E. B., and Williams, R. R., *Philippine J. Sc.*, 1913, viii, 180.
[10] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 88.
[11] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 432.
[12] Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, xxxvii, 201.

Seidell[11] for the vitamine. There seems no doubt from our experiments that this material when acted on by acid or alkali is changed (presumably by hydrolysis) to a form which is more immediately available for the yeast. The treatment does not change the total amount of yeast which is produced in 2 or 3 days but makes the initial growth for 24 hours as much as three times as fast. These results are in accordance with the observations mentioned above on the supposed hydrolysis. This, it is thought, adequately explains the result obtained in the experiment on phosphotungstic acid precipitation as both the "filtrate"

TABLE XIII.

	Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 25 cc. H_2O	25	3	6	4	4.7
II.	25 cc. control solution + 1 cc. H_2O + 1 cc. ether extract of egg yolk	17	5	14	7	9
III.	25 cc. control solution + 1 cc. H_2O + 1 cc. alcoholic extract of egg yolk	29	15	120	29	68
IV.	25 cc. control solution + 1 cc. ether extract of egg yolk + 1 cc. alcoholic extract of egg yolk	26	20	200	23	59

and "decomposed phosphotungstates" had stood in 5 per cent H_2SO_4 for some time.

The results obtained on various other preparations would suggest that the substance promoting the growth of yeast, as it exists in yeast and wheat germ (alcoholic extracts), is not very unstable in the presence of alkali. However, a previous exhaustive extraction with dry ether seems to render the alcoholic extract obtained afterward more labile to alkali. We have some evidence that this is true also of the fullers' earth preparation. It seems to be unstable to alkali when the material is extracted with ether before alcoholic extraction. This will be referred to later.

XI. Fat-Soluble Vitamine.—It was thought desirable to find out whether the fat-soluble vitamine has any effect on yeast growth.

The yolk of hard boiled egg was selected as a source as it contains both water-soluble and fat-soluble vitamines in good quantity. The yolk was removed from a hard boiled egg, partially dried in a desiccator, then ground with plaster of Paris, and thoroughly dried over calcium chloride in a vacuum desiccator. The material was continuously extracted for 10 hours with sodium-dried ether, taking extra precaution to avoid the presence of moisture. It was then extracted for 3 hours with 95 per cent alcohol. Each extract was evaporated to dryness, digested as completely as possible in water, and filtered. The solutions were made so that 1 cc. was equivalent to 0.1 gm. of dry egg yolk.

The results obtained after 22 hours growth are given in Table XIII. The effect of the fat-soluble vitamine, if there is any effect, is not marked either as a substitute for the alcohol-soluble substance, or as complementary to it. If the yeast has a requirement for the fat-soluble vitamine it is exceedingly minute.

DISCUSSION.

In the preceding experiments it has been shown that the substance promoting growth of yeast occurs in the same materials as those in which vitamine has been found; namely, protein-free milk, wheat germ, lactose, yeast, egg yolk, and pancreatin. The substance is none of the commoner amino-acids contained in an acid digest of casein with tryptophane added. It has the same properties of solubility, precipitation by phosphotungstic acid, adsorption in fullers' earth, heat stability, and behavior toward acid and alkali, as nearly as we know those properties, as the water-soluble vitamine, and in addition the two substances so far as known have no divergent properties. A warning should be given that the results reported here in different experiments are not comparable one with another and were *not* designed to show quantitative comparisons of different materials. As far as we are able to judge, however, taking into consideration the apparent hydrolysis factor which probably is important, as well

as the method of extraction, those substances which are richest in vitamine are richest in the yeast growth substance. The fullers' earth preparation is richest of any of the sources tested. Wheat germ and yeast are poorer than the fullers' earth preparation and are of the same order as has been found to be true of the vitamine content in animal experiments. Milk is still poorer in the yeast growth substance as is also true of its vitamine content.

From the cumulative evidence offered we believe we are justified in concluding that as far as present knowledge is concerned the substance or substances which stimulate the growth of yeast is or are identical with the substance or substances which in animal nutrition prevent beri-beri or polyneuritis.

If this conclusion is true the water-soluble vitamine must be a most fundamental nutritional requirement playing an indispensable rôle for a great variety of organisms. It has not before been shown conclusively to be necessary for the nutrition of any members of the plant kingdom. Some suggestions have been made as to its possible importance in the nutrition of bacteria. We have accidentally observed that some species of molds are apparently able to produce it. Pacini and Russell[13] have shown that typhoid bacilli must be able to produce it.

As it is apparently possible to cause a single yeast cell to produce from 20 to several thousand cells in 24 hours by varying the vitamine content of the culture medium, we hope the method may be valuable both as a qualitative and ultimately as a quantitative test for vitamine. Work is now being done to make the test more applicable quantitatively and will be reported upon later. As a qualitative test extremely small amounts may be detected. The most serious obstacle in the purification and study of vitamines has been the difficulty of obtaining sufficient material to work with and test. Yeast can be used to test for the water-soluble vitamine and only infinitesimally small amounts need be used for testing. The accompanying photomicrographs (Figs. 1 and 2) show the effect of the addition of 0.5 mg. of actual crude material to about 30 cc. of control solution and subsequent incubation for 24 hours. The drops in which the cells were growing weighed approximately 0.03 mg. each and contained therefore

[13] Pacini, A. J. P., and Russell, D. W., *J. Biol. Chem.*, 1918, xxxiv, 43.

about one millionth of the 0.5 mg. of total material added. The material added was crude, being obtained simply by adsorption on fullers' earth from a malt wort and subsequent extraction with alkali. The test can be easily applied after a little practice. The vitamine if present in sufficient but very small quantity can be detected in the course of 4 or 5 hours.

Already the method has contributed something in the way of additional information in connection with the apparent hydrolysis for which the evidence is now more certain.

It seems that we have at least a partial explanation for the divergence of results in stability of the vitamine toward alkali, in the effect of previous ether extraction. In recent work McCollum and Simmonds[10] found the vitamine to be very unstable toward alkali. The material they used was previously extracted for 18 hours with ether. Voegtlin and Lake[14] observed that meat which had been freed from fat lost its antineuritic properties under the influence of heat and alkali much more easily than meat with the fat present.

The difficulty of obtaining pure materials and the likelihood of wrong inferences drawn from work with impure material is emphasized by our work. In earlier work on yeast, yeast no doubt grew better in "synthetic" media than it would have if the asparagine, ammonium tartrate, sugar, etc. used had been purer. Devloo's results on the preparation of "bios" from lecithin parallel the findings of an early investigator who cured polyneuritis on "pure" lecithin.

At this point I wish to thank Professor F. C. Koch for helpful suggestions and interest throughout the work and for his generosity in providing equipment.

CONCLUSIONS.

1. A substance of unknown nature, which is a constituent of yeast, is necessary in addition to the ordinary nutrients for the nutrition of yeast cells.

2. This substance (or substances) based upon identical occurrence and various properties is concluded to be identical with the beri-beri-preventing vitamine.

[14] Voegtlin, C., and Lake, G. C., *Am. J. Physiol.*, 1918–19, xlvii, 558.

3. The fat-soluble vitamine apparently has no effect on yeast growth.

4. The growth of single cells of yeast may be used as a simple biological test for vitamine, and it is hoped may be used to advantage in quantitative studies.

EXPLANATION OF PLATE.

FIG. 1. Growth produced by a single cell in 24 hours in "synthetic" solution.

FIG. 2. Growth produced in 24 hours under identical conditions except for the addition of one part in 60,000 of crude vitamine-containing material (cf. p. 484).

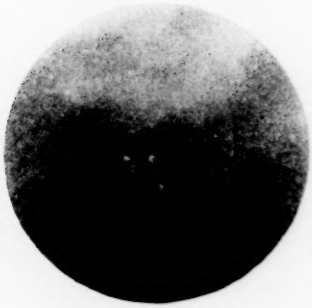

FIG. 1.

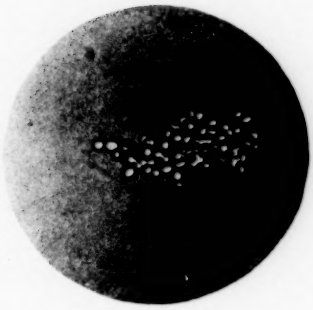

FIG. 2.

RELATIONSHIP OF THE PANCREATIC ENZYMES.

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Our idea in presenting this paper is not so much that the basic facts are new as that there is a real need for exact data on mixtures such as we have described. No comparative study of the various enzymes of the pancreas in relation to each other has been made with samples of known origin and composition. The methods used in preparing these samples are simple, practical, and capable of rendering representative mixtures which approach conditions existing in the body.

Attempts were also made to increase the activity of these enzymes to a maximum by means of sodium chloride, duodenal tissue, and fresh bile. Sodium chloride was employed because we have found it the most satisfactory means of preventing loss of diastasic power in the pancreas while drying. The duodenal mucosa and the bile are close associates of the pancreatic secretion and are generally considered activators of the proteolytic and lipolytic enzymes, respectively.

Starting then from these well known points, the relationship of the enzymes with and without activation becomes of interest. For control purposes, whole glands as well as duodenal tissue and fresh bile were prepared in additon to the mixtures, and tested along with the other samples.

Hog glands were selected because the omnivorous hog shows closer digestive relationship to the human race than do herbivorous animals such as cattle and sheep, and also because the large majority of commercial pancreatic preparations are derived from the hog. The glands were taken at random from the Chicago abattoir and represent hogs raised in the middle west. The pancreases, duodenums, and bile were collected from separate animals.

The duodenal tissue from hogs is always acid in reaction, while the bile is neutral or slightly alkaline. All the pancreatic glands which have come under observation in this laboratory during the past 8 years, amounting to several hundred lots and representing millions of hogs, were acid to litmus. The same is true of the cattle and sheep pancreases examined. It is certain, therefore, that the normal reaction of the pancreas is acid. Attention has been called to this fact in earlier communications[1] and the present statements may be regarded as additional and conclusive evidence.

The entire pancreatic glands were carefully removed immediately after slaughter of the animals, trimmed free from connective and other adherent tissue, and finely minced. A dozen or more of the pancreases from each lot were transferred, while still retaining the animal heat, to the vacuum oven and desiccated whole without disturbing the fatty tissue and protecting membranes surrounding the gland itself. This was done to eliminate the disturbing action of mincing and to minimize the possibility of atmospheric influence. These glands, therefore, represent very closely the actual condition existing in the pancreas of the living animal.

The duodenums, consisting of the first 12 inches of the small intestines immediately below the pyloric valve, were likewise removed and freed from adipose and connective tissue, care being taken to discard all traces of pancreatic structure. The tubes were cleansed by means of running water, before mincing. It may be mentioned here that in hogs the opening of the bile duct is about 2 inches and that of the pancreatic duct 4 to 5 inches below the pylorus. The bile was obtained directly from the gall bladders. In each set of experiments the mixtures were made from the same batch of minced material by weighing out the ingredients and mixing thoroughly, first by hand and then by running the mixture through a small meat chopper. The various preparations were spread on agate trays each holding 200 gm. or more and dried *in vacuo* at temperatures ranging from 30–40°C. After desiccation, the material was extracted with petroleum ether in Soxhlet extractors and powdered to 60 mesh.

[1] Long, J. H., and Fenger, F., *J. Am. Chem. Soc.*, 1915, xxxvii, 2213; *ibid.*, 1916, xxxviii, 1115.

In the tabulations will be found the date of collection and the number of glands and other material employed; also the loss in moisture, petroleum ether-soluble material, and the yield of desiccated fat-free substance. All enzymic determinations were made on the powdered samples.

Methods.

The methods employed in measuring the activity of the samples were mainly of a routine character. Strict care was taken to make them uniform so that in every case measurement of activity was comparable.

The diastasic activity was a measure of the gm. of anhydrous potato starch which 1 gm. of enzymic powder will change to dextrin, colorless to iodine, in 10 minutes at 40°C. The method was that outlined by Johnson,[2] and the reported values are in all cases the mean of at least three independent and closely agreeing checks on the sample.

The fat-splitting activity was measured by the cc. of 0.10 N NaOH required to neutralize the acid formed from the action of 0.100 gm. of the enzymic powder on 10 cc. of emulsified olive oil. The emulsion was formed by neutralizing the oil to phenolphthalein with 0.25 N NaOH. When a large enough quantity of oil for each series is taken and the mixture with NaOH is thoroughly shaken, an emulsion is formed which holds very well and by neutralizing the whole quantity for a series, a uniform sample is obtained.

The mixture of powder and oil was digested for 2 hours at 40°C. in a water bath, shaking every 15 minutes. At the end of digestion, 25 cc. of neutral alcohol and 5 cc. of acid-free ether were added and the mixture titrated with 0.10 N NaOH using phenolphthalein as indicator. For control the same procedure was followed except that the enzyme was killed by boiling the powder in a minimum amount of water for 15 minutes. In every case correction for this blank has been made. This method has been described by Wohlgemuth.[3] The action of lipase on olive oil seems to be representative of this group of ester-splitting ferments.

[2] Johnson, W. A., *J. Am. Chem. Soc.*, 1908, xxx, 798.

[3] Wohlgemuth, J., Grundriss der Fermentmethoden, Berlin, 1913, 102.

Two methods of measuring tryptic activity were used. One, the well known Fuld-Gross method, needs no outlining in these pages, while the other is almost as familiar. This second is that of measuring the amino-acids formed by digestion with the enzyme. It having been found by one of us[4] that the optimal activity of trypsin is to be obtained in digestion of that enzyme with blood fibrin in a mixture of di- and monosodium phosphate, this mixture was used as follows: 900 cc. of 0.067 M disodium phosphate and 100 cc. of 0.067 M monosodium phosphate (prepared according to Sörensen[5]) were mixed and 25 cc. of this were taken for each digestion, mixed with a weight of dried 40 mesh blood fibrin, equivalent to 1 gm. of protein, and 25 cc. of water. This mixture was digested for 3 hours at 40°C., shaking every 15 minutes.

After digestion each mixture was treated with 1 gm. of barium chloride and barium hydroxide to strong alkalinity (approximately 20 cc. of saturated Ba $(OH)_2$), made to 100 cc. and after the precipitated proteins and phosphate had settled, a 50 cc. aliquot was filtered off, made neutral to litmus, 10 cc. of neutral formaldehyde were added, and the mixture was titrated with 0.25 N NaOH using phenolphthalein as indicator. This titration represents, when corrected for its control, the equivalent of mono-amino-acid nitrogen formed in one-half of the digestion.

The controls here, as in other cases, consist in parallel determinations in which the enzyme was "killed" by boiling 15 minutes. These blanks were very small, in no case amounting to more than 1.2 cc. of 0.25 N NaOH, an average value being 1.0 cc., and represent both the action of the buffer solution on the proteins and the acidity of and amount of amino-acid present in the enzymic powder itself. In each case the values quoted in Table I have been corrected for this blank and represent real enzymic activity.

The fibrin used in these experiments was prepared in this laboratory from fresh beef blood. The casein for the Fuld-Gross tests was prepared by one of us from fresh, commercial fat-free milk by a series of precipitations until free from salts. The purified precipitate was washed rapidly with alcohol and ether and powdered to 40 mesh.

[4] Long, J. H., and Hull, M., *J. Am. Chem. Soc.*, 1917, xxxix, 1051.
[5] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.

All the samples prepared for this series of experiments were tested according to the well known colorimetric method of measuring pH values. 0.10 gm. of the powders was mixed intimately with 30 cc. of CO_2-free water and filtered after standing 15 minutes. 10 cc. of this filtrate were used for the determination, with neutral red and *p*-nitrophenol as indicators. Mixtures of 0.067 M mono- and disodium phosphate were used as standards. The straight pancreas samples showed pH values ranging between 6.11 and 6.14 while that of the duodenal tissue was found to be 6.21. We were unable to make an accurate determination of the bile, due to its strong color which masks the reaction. It has been shown by one of us,[1] however, that the pH value for bile when measured by the potential cell, lies between 7.04 and 7.21. The reaction consequently is neutral or very faintly alkaline. All the mixtures in the tabulation, including those containing bile, showed pH values between the limits of 6.11 and 6.21, and are, therefore, acid in reaction.

Tests were also made on the reaction of the buffer solution used in the fibrin digestion experiments with trypsin. The two phosphate solutions after mixing and dilution with water to 50 cc. showed a pH value of 8.02. When this same dilution was mixed with enzymic powder and fibrin exactly as for digestion and due time allowed for the establishment of equilibrium, the pH value of the filtrate was found to be 7.40. The pH value of the liquids after digestion for 3 hours at 40°C. ranged from 7.30 for straight pancreas powder, to 7.18 in cases where the pancreas and duodenal mixtures were employed. All the other samples come within these two limits. The alkalinity of the solution naturally decreases inversely to the activity of the proteolytic enzyme and the amounts of amino-acids formed during digestion.

Tabulation.

It will be seen from the tabulated results that the whole normal pancreas possesses high diastasic, considerable lipolytic, and some proteolytic power. The diastase is present in fully activated form ready for immediate use, while the lipase and trypsin need the stimulating effect of the bile and duodenal mucosa respectively, before reaching maximum activity.

Both mincing and drying lower the diastasic power of the fresh gland materially, while the proteolytic and lipolytic enzymes seem more resistant to mechanical interference. The quicker the moisture is removed from the minced material the smaller will be the loss in diastase. Thus, vacuum drying at blood heat is less destructive than desiccation under atmospheric pressure, and especially at higher temperatures. Drying in air, for instance, at 50°C. reduces the diastase power still further, indicating the sensitiveness of the enzyme to heat.

The mincing process breaks up many of the pancreatic cells, causing the liquid or semiliquid contents to be mixed. This radical treatment completely upsets the glandular equilibrium and it is only logical to expect enzymic disturbances to occur. The diastase, being the most sensitive of the enzymes, naturally suffers the most. If the gland is left intact and dried *in vacuo* at low temperature, the equilibrium is not disturbed and [illegible]nzymic deterioration is evidenced.

The loss in diastasic activity may be prevented by the addition of a small amount of sodium chloride, preferably in combination with bile, to the freshly minced material before desiccation. This also applies to pancreatic mixtures containing duodenal mucosa and activated trypsin. The influence of the salt is of a protective or stabilizing nature rather than a true activation process, since the diastasic power is not increased materially beyond that of the undisturbed whole gland. Sodium chloride seems to have a slightly stimulating effect on the lipolytic enzyme also, while it does not influence the trypsin one way or the other.

In the living animal, the stabilizing action of sodium chloride is apparently not needed until the pancreatic juice enters the duodenum. It has been shown in an earlier paper that the juice obtained from the entire fresh hog pancreas by centrifugal force contains only 0.012 per cent chlorine, equivalent to 0.02 per cent sodium chloride. When allowance is made for the salt present in the natural blood supply of the pancreas, it will be seen that the true pancreatic secretion is practically free from chlorides. The sodium chloride is encountered in the duodenum where it is present as a natural constituent of the bile. The samples of bladder bile employed in these experiments contained on an average 0.33 per cent of sodium chloride. Since, in this case, the bile duct enters

the duodenum above the pancreatic outlet, the presence of sodium chloride is always insured during active digestion. The hydrochloric acid present in the gastric content gradually combines with the organic sodium salts in the bile, furnishing additional sodium chloride which likewise tends to maintain the diastasic power. The results shown in Experiment 4 indicate that the sodium chloride as present in the bile is not sufficiently available to completely offset loss in the diastasic power during the desiccation processes. The mucin of the bile evidently retards the action of sodium chloride to a considerable extent. If a little salt is added to the pancreas and bile mixture, as in Experiment 5, there is a small increase in diastasic action even over that present in the whole gland.

In Experiment 8 we have a condition which comes within reach of that actually existing in the animal, namely a mixture of pancreatic secretion with duodenal mucosa and bile. As a consequence all three enzymes occur in perfect harmony and fully activated without causing interfering disturbances. Under such conditions, the addition of sodium chloride does not produce further increases in the diastasic activity as Experiment 9 indicates. The importance of bile and duodenal mucosa for the proper digestion of food and the serious consequences resulting from lack of either or both, are obvious.

The diastasic enzyme is very sensitive to acids and alkalies. Addition of 0.1 per cent of concentrated hydrochloric acid to the minced pancreas lowers the diastase materially, while 1.0 per cent practically destroys it. The proteolytic power is increased somewhat by this acid treatment but not nearly as pronouncedly as by duodenal mucosa. Sodium bicarbonate lowers enzymic action gradually as the acidity of the gland decreases. It requires approximately 2.5 per cent of this salt to bring the fresh gland to litmus neutrality. On the alkaline side of neutrality all three enzymes become inert. Even distilled water depresses the enzymes. 10 per cent of distilled water added to the fresh gland reduces the diastasic activity very materially and renders the proteolytic enzyme practically inert, while the lipase is but slightly affected. Here again, sodium chloride protects the diastasic enzyme to a considerable extent.

All the pancreases examined show some degree of proteolytic power and we have been unable to locate a single sample of known source which was inert in this respect. This is shown further by the self-digesting properties of the pancreas. A number of whole glands, immediately after removal from the animals, were placed on trays kept at 50°C. under ordinary atmospheric conditions. In the course of 2 hours the glands became very soft as self-digestion rapidly took place. The fat and membranes surrounding the fresh glands formed a protective coating which held the individual gland together and prevented running. The heating was continued for 15 hours. Upon examination the interiors of the glands were found to be completely digested and still moist and soft. Many clusters of amino-acid crystals were encountered throughout the mass, indicating that the digestion was carried well into the amino-acid stage. The soft mass was spread thin on trays and dried at the same temperature. The fat-free powdered sample still showed slight proteolytic and lipolytic activities while the diastasic properties were completely lost.

The addition of 25 per cent of duodenal tissue to freshly minced pancreases increases the proteolytic power approximately fourfold over that present in the original whole gland when measured on sodium caseinate by the Fuld-Gross method, and about three times when blood fibrin and Sörensen's formol titration are employed. This discrepancy is readily accounted for when it is considered that we are dealing with two distinctly different proteins and measuring different stages of hydrolysis.

Preliminary experiments not included in the present tabulation were conducted to determine the amount of fresh duodenal tissue necessary to activate the trypsin to a maximum. Admixtures of 5, 10, 15, 20, and 25 per cent of duodenums to the pancreases were tried. While these mixtures showed increasing activity of the proteolytic enzyme they also furnished evidence of inversely proportioned decreases in diastasic and lipolytic strength.

By referring to the tabulated results it will be seen that the depressing action of duodenal mucosa on the other two enzymes is very marked in the absence of bile. The presence of bile protects the diastase and renders the lipase immune to tryptic destruction. The duodenal tissue as obtained from the healthy living animals seems to be fully sensitized for tryptic activation purposes and it

has been our experience that the addition of either hydrochloric acid or sodium carbonate or bicarbonate does not stimulate but rather tends to lower its efficiency. The activation takes place on the acid side of neutrality since both the pancreas and duodenal tissue are acid in reaction.

In another series of preliminary experiments not tabulated here it was found that small amounts (1 per cent) of the powdered 75: 25 pancreas and duodenum mixture, when added to freshly minced hog pancreases, activate the proteolytic enzyme quite satisfactorily. If these mixtures, however, are employed in the same proportions for activation of new lots of glands, the results are much lower and it becomes necessary to use a higher percentage to produce the same effect. This shows plainly that tryptic activation is due to some substance present in the duodenum, with a specific but limited power of activation, and not to the activated proteolytic enzyme acting as a catalyzer. These samples also showed that the diastasic enzyme is but slightly affected by the actual tryptic activation process while the lipolytic activity is depressed materially. That it requires 25 per cent by weight of fresh duodenal tissue and only 1 per cent of the dry fat-free pancreas and duodenum mixture to produce the same tryptic activity, is readily conceivable when the general retarding nature of moist mucus is considered.

The maximum digestive power of the proteolytic enzyme is not reached at body temperature. This applies to the enzyme as it occurs in the pancreas as well as after it has been reinforced with duodenal mucosa. Fibrin digestion experiments were carried out as outlined above on representative and identical samples at 40 and also at 50°C. The six selected samples titrated 7.1, 7.2, 14.7, 16.4, 16.8, and 21.7 cc. of 0.25 N NaOH after digestion at 40°C. and 14.6 16.0, 28.2, 31.9, 32.8, and 45.8 cc. of 0.25 N NaOH at 50°C. An actual increase of 100 per cent in the amino-acid nitrogen is obtained by raising the digestion temperature from 40 to 50°C. The importance of accurate control of time and temperature factors becomes very apparent when these results are borne in mind.

When liquid bile is mixed with freshly minced pancreases, the power of the lipolytic enzyme is almost doubled. Besides this activation property, the function of the bile seems to be that of protecting the fat-splitting process from tryptic interference. In

TABLE I.

Experiment No.	Composition of samples.	Date of collection and preparation.	Number of glands.	Fresh material. Moisture on fresh material.	Fresh material. Petroleum ether-soluble material.	Fresh material. Desiccated fat-free material.	Desiccated fat-free material. Diastasic power.	Desiccated fat-free material. Proteolytic power. Fuld-Gross method.	Desiccated fat-free material. Proteolytic power. Sörensen formol titration 0.25 N NaOH per gm. of protein (0.175 gm. N.).	Desiccated fat-free material. Lipolytic power. 0.10 N NaOH required to neutralize 10 cc. neutral olive oil emulsion, 0.1 gm. enzyme.
		1919		*per cent*	*per cent*	*per cent*			*cc.*	*cc.*
1.	Entire hog pancreases desiccated whole.	Feb. 4	12	62.0	20.4	17.6	1:91	1:27	6.4	33.8
		" 6	16	62.0	19.4	18.6	1:95	1:16	7.2	34.5
		" 19	17	58.0	22.2	19.8	1:87	1:28	7.2	22.8
		March 4	15	60.4	20.7	18.9	1:87	1:17	7.6	20.4
		" 12	15	61.0	18.0	21.0	1:100	1:20	7.1	20.6
	Average.		75	60.7	20.1	19.2	1:92	1:22	7.1	26.4
2.	Minced hog pancreases.	Feb. 4	60	62.0	18.8	19.2	1:62	1:19	7.1	24.4
		" 19	50	60.2	18.2	21.6	1:57	1:24	7.8	24.8
		March 4	50	63.5	17.7	18.8	1:49	1:20	7.4	27.5
		" 12	50	60.5	18.0	21.5	1:60	1:19	6.8	21.4
		April 1	50	56.0	26.5	17.5	1:50	1:19	7.3	23.2
	Average.		260	60.5	19.8	19.7	1:56	1:20	7.3	24.3
3.	Minced hog pancreases, 99.0 per cent. Sodium Chloride, 1.0 " "	Feb. 4	60	61.5	18.0	20.5	1:100	1:11	6.7	30.5
		" 19	50	59.8	18.2	22.0	1:83	1:28	7.1	26.1
		March 4	50	63.0	17.3	19.7	1:100	1:18	6.4	29.0
		April 1	50	56.0	26.1	17.9	1:87	1:17	6.9	26.7
	Average.		210	60.1	19.9	20.0	1:92	1:18	6.8	28.1

4.	Minced hog pancreases, 90.0 per cent.	Feb. 19	50	63.3	17.0	19.7	1:51	1:15	7.4	45.3
	Liquid hog bile, 10.0 " "	March 1	50	60.0	22.4	17.6	1:61	1:16	7.4	45.0
	Average.		100	61.7	19.7	18.6	1:56	1:15	7.4	45.2
4a.	Minced hog pancreases, 90.0 per cent.	March 4	50	66.0	16.1	17.9	1:25	1:0.5	2.4	28.5
	Distilled water, 10.0 " "									
5.	Minced hog pancreases, 89.0 per cent.	Feb. 19	50	62.5	16.4	21.1	1:105	1:14	7.6	44.9
	Liquid hog bile, 10.0 " "	March 4	50	63.0	16.9	20.1	1:86	1:17	7.1	41.2
	Sodium chloride, 1.0 " "	April 1	50	59.0	24.2	16.8	1:105	1:17	7.4	41.0
	Average.		150	61.5	19.2	19.3	1:99	1:16	7.4	42.4
5a.	Minced hog pancreases, 89.0 per cent.	March 4	50	66.0	15.8	18.2	1:59	1:2	3.1	29.1
	Distilled water, 10.0 " "									
	Sodium chloride, 1.0 " "									
6.		Feb. 4	60							
	Minced hog pancreases, 75.0 per cent.		30	65.0	15.7	19.3	1:35	1:100	16.6	7.8
	" " duodenums, 25.0 " "	" 19	50							
			30	65.0	15.0	20.0	1:24	1:117	17.4	12.5
	Average.		110	65.0	15.3	19.7	1:30	1:109	17.0	10.2
7.	Minced hog pancreases, 74.0 per cent.	Feb. 4	60	63.5	15.6	20.9	1:57	1:89	16.8	8.8
	" " duodenums, 25.0 " "	" 19	50	64.5	14.4	21.1	1:53	1:111	16.8	12.5
	Sodium chloride, 1.0 " "									
	Average.		110	64.0	15.0	21.0	1:55	1:100	16.8	10.6

TABLE I—*Concluded.*

Experiment No.	Composition of samples.	Date of collection and preparation.	Number of glands.	Fresh material. Moisture on fresh material.	Fresh material. Petroleum ether-soluble material.	Fresh material. Desiccated fat-free material.	Desiccated fat-free material. Diastasic power.	Desiccated fat-free material. Proteolytic power. Fuld-Gross method.	Desiccated fat-free material. Proteolytic power. Sörensen formol titration 0.25 N NaOH per gm. of protein (0.173 gm. N.).	Desiccated fat-free material. Lipolytic power. 0.10 N NaOH required to neutralize 10 cc. neutral olive oil emulsion, gm. enzyme.
				per cent	*per cent*	*per cent*			*cc.*	*cc.*
8.	Minced hog pancreases, 65.0 per cent. " " duodenums, 25.0 " " Liquid hog bile, 10.0 " "	March 12 April 1	50 50	70.0 66.5	12.1 18.6	17.9 14.9	1:63 1:49	1:111 1:105	17.6 14.9	38.6 40.0
	Average.		100	68.2	15.4	16.4	1:56	1:108	16.3	39.3
9.	Minced hog pancreases, 64.0 per cent. " " duodenums, 25.0 " " Liquid hog bile, 10.0 " " Sodium chloride, 1.0 " "	Feb. 9	50	66.8	13.2	20.0	1:50	1:111	18.0	40.2
10.	Hog duodenums minced.	Feb. 4 " 19	30 30	80.0 82.0	2.5 2.8	17.5 15.2	Negative. "	Negative. "	Negative. "	Negative. "
	Average.		60	81.0	2.6	16.4				
1.	Fresh hog bile.	Feb. 19 March 12	50 50	86.0 88.6	0.5 0.2	13.5 11.2	Negative. "	Negative. "	Negative. "	Negative. "
	Average.		100	87.3	0.4	12.3				

combination with duodenal mucosa, bile prevents the deterioration of the sensitive diastasic enzyme. This action seems to be due principally to the sodium chloride present in the bile.

The chlorides present in hog bladder bile vary somewhat, due to local conditions and kind of food ingested. Since sodium salts predominate in bile most of the chlorine is present as sodium chloride. We found a minimum of 0.17 per cent and a maximum of 0.22 per cent of chlorine, equivalent to 0.28 per cent and 0.37 per cent of sodium chloride respectively, in the various samples of liquid bile employed in these experiments.

We are greatly indebted to Mr. Paul Rudnick, Chief Chemist of Armour & Company, for valuable assistance and courtesies which made this investigation possible. We also wish to express our thanks to Mr. Joseph Brunn for help in collecting the material and preparing the many samples.

SUMMARY.

In conclusion we wish to state that while the basic facts concerning the various enzymes of the pancreas, set forth here, are not materially new, they present, however, some points of interest.

1. The data obtained on the whole pancreas represent as nearly as possible the actual conditions existing in the living animal. The disturbance of glandular equilibrium necessarily resulting from mincing and mixing with consequent liberation of the cell contents, autolysis, etc., has been practically avoided, or at least materially minimized, by drying the entire gland *in vacuo*.

2. The normal pancreas as removed from the animal is of distinctly acid reaction and possesses high diastasic, considerable lipolytic, and some proteolytic activity. The diastasic enzyme is present in fully activated form while the strength of the lipolytic enzyme is almost doubled by addition of the bile. The proteolytic enzyme is activated to several times its original strength by duodenal mucosa.

3. Bile was found to be void of any measurable enzyme action. Besides its well known function of augmenting the activity of the lipolytic enzyme, it seems to protect the fat-splitting process against tryptic interference. Combined with duodenal mucosa, it stabilizes the otherwise sensitive diastasic enzyme.

4. It is possible to produce and maintain maximum activity of the three enzymes in the removed pancreas, without causing interfering disturbances, by the addition of adequate amounts of bile and duodenal mucosa.

5. Certain characteristic behaviors and interrelations of the enzymes in given samples are brought out.

STUDIES OF THE CONCENTRATION OF CATALASE IN URINE, CHYME, AND FECES.

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(Received for publication, May 21, 1919.)

Estimation of Catalase.

When a solution of catalase is added to a solution of hydrogen peroxide, decomposition of the hydrogen peroxide takes place if the reaction of the mixture is not too acid. By the decomposition water and molecular oxygen are produced. The volume of oxygen is a measure of the extent of the decomposition. Coincidently with this process a destruction of the catalase takes place, if the hydrogen peroxide solution is not too weak. Consequently the reaction, $2\ H_2O_2 = 2H_2 + O_2$, is finished within a limited time.

The pyometer, or apparatus for measurement of the oxygen, which we have used[1] consists of a 40 to 60 cm. glass tube, which is bent as shown in Fig. 1—two parallel tubes connected by an M-shaped middle piece. One of the tubes, the measuring tube, is long (20 cm.) and is provided with a graduation. The other is short (5 to 8 cm.) and provided with a piece of rubber tubing about 2 cm. long (Fig. 1). If the free end of the rubber tubing is pinched together, the short limb of the pyometer is shut, and by so doing one may adjust the column of liquid in the pyometer. The internal diameter of the tube is 0.5 cm.

Principle of the Method.—The principle of the method is that a measured quantity of the solution of catalase is placed in the pyometer, which is filled with H_2O_2, and is confined in it. The catalase will decompose a certain quantity of H_2O_2. The liberated oxygen depresses the liquid in the short limb of the pyometer

[1] The apparatus may be purchased from the Emil Greiner Co., 55 Fulton Street, New York.

and raises the column of hydrogen peroxide in the measuring tube to an extent which indicates the volume of oxygen evolved.

Determination.—From a burette or a pipette as much 3 per cent hydrogen peroxide is run into the pyometer through the measuring tube as is necessary to fill the M-tube and the lower parts of the two vertical tubes. Then 0.2 cc. of the catalase solution is added by letting it run from a pipette through the rubber tubing down into the H_2O_2 in the short limb. The inside of the rubber tubing is washed with two to three drops of H_2O_2, which one lets run down into the pyometer. Then the pyometer is placed at such an angle that the surface of the liquid in the measuring tube stands just at the height of the zero point. While the pyometer is held in this position, a pinch-cock is placed on the free end of the rubber tubing, which is closed by this means. The apparatus is placed in the stand.

The oxygen liberated is received partly in the short limb, partly in the nearer curve; if much oxygen is developed some will also pass over into the other curve. The displacement of the liquid in the measuring tube indicates the volume of liberated oxygen. The process is finished after 3 to 24 hours, most quickly when smaller concentrations of catalase are present.

Hydrogen Peroxide Unit of Measurement (HPU).—As unit of measurement is used the proportion between the volume of oxygen developed and the volume of catalase solution which caused it.

0.2 cc. of a liquid in a tube with an internal diameter of 0.505 cm. will fill a space 1 cm. long. Therefore if one is measuring the decomposition, which is brought about by 0.2 cc. of a solution of catalase, and finds that the surface of the liquid in the measuring tube is raised n cm., there will be developed n times as much oxygen as the volume of added solution of catalase run in. In this paper the definition of the number of units of the catalase solution therefore is n. This unit is called the "hydrogen peroxide unit" (HPU).

Sources of Error.—The reaction between catalase and H_2O_2 ought to take place in a moderately acid medium. The presence of alkalies will cause non-specific decompositions. Mineral acids prevent or diminish the process, even when they are very much diluted. It is necessary to be sure that the 3 per cent solution of

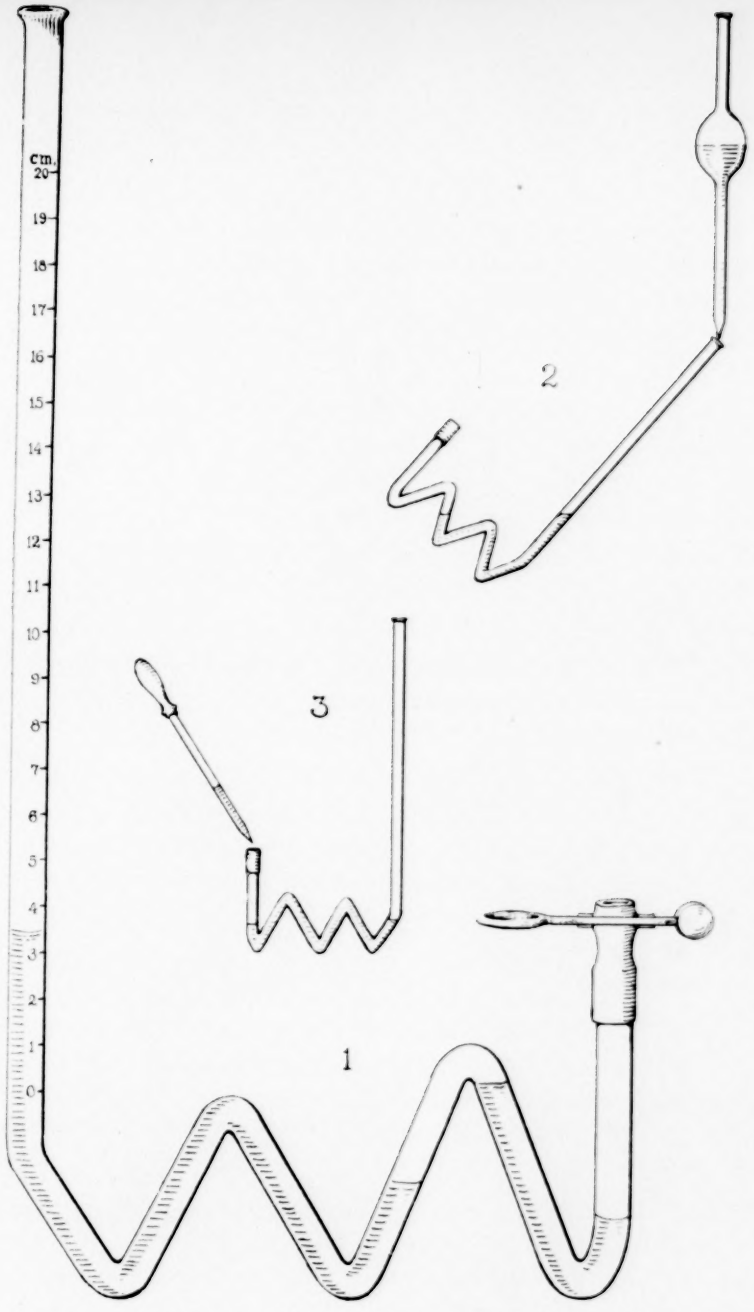

FIG. 1. 1. Pyometer in action. 2. Addition of peroxide. 3. Addition of catalase solution.

hydrogen peroxide is neither too acid nor is decomposed spontaneously by standing.

For doing away with an alkaline reaction I add small amounts of boric acid crystals to the solution of catalase.

The determination is carried out at room temperature, but the reacting solution must be protected from direct sunlight. It is emptied by pouring the contents out through the short limb, which by that means is rinsed. Further cleaning is seldom necessary.

Estimation of Catalase in Urine.

Demonstration of pus in urine is possible by (1) examination with an alkali (Donner), (2) microscopy, and (3) reaction with hydrogen peroxide. The first of these methods is not suitable for quantitative use, the second is troublesome and cannot be used when the pus corpuscles are disintegrated, while by the third method quantitative determination is easily performed suitable for clinical use.

Determination of Catalase in Urine.—The urine is acidulated as soon as possible with boric acid crystals, as it is important that the free ammonia of the urine be neutralized. After that 0.2 cc. of urine is introduced into the pyometer, which is filled in advance with hydrogen peroxide through the long limb. The urine is added through the short limb, which is washed with three drops of hydrogen peroxide; the surface of the liquid in the measuring tube is brought to the zero point of the graduation; the pinchcock is put on and the pyometer is placed in a shady place for a definite period.

Acidity of the Urine.—The catalase reaction takes place most quickly in a neutral or a very moderately acid medium. With more acid reaction the process is retarded and it is prevented more or less completely by a strongly acid reaction. The highly dissociated acids (HCl, H_2SO_4, etc.) have an inhibitive influence; while the slightly dissociated acids have a smaller influence on the process, because they do not remove the hydrogen ion concentration so far from the neutral point. Such acids are, for instance, uric acid and boric acid. The latter even in half saturated solution does not affect the influence of the catalase appreciably. If the medium is on the alkaline side of the neutral

point, non-specific decomposition of hydrogen peroxide may occur, because small amounts of alkali (ammonia, carbonate) will decompose H_2O_2. If the mixture is very strongly alkaline, the process is totally prevented, even if catalase is present. Before the urine is used for examination in the pyometer, one must therefore test the reaction to litmus paper. If it is not acid, it should be acidulated with boric acid crystals.

Catalase in Urine.—Catalase in urine may originate from pus cells, from red blood corpuscles, from epithelial cells, from casts of the urinary tubules, from bacteria, and from alkaline products. The catalase content of casts of the urinary tubules and of the epithelial cells is quantitatively of secondary importance. *Bacillus coli* is very deficient in catalase, while the urea-decomposing bacteria produce substances that give a non-specific decomposition which may be prevented by admixture of boric acid. The remaining sources of catalase are then red and white blood corpuscles. A distinction between the catalase which originates in the red and that which originates in the white blood corpuscles is impossible. On the average a white blood corpuscle contains twice as much catalase as a red one. If red and white blood corpuscles are found together, the estimation described indicates the sum of the catalase from the leucocytes and that from the erythrocytes.

Effect of Urinary Sediments.—If the urine sample is quite fresh, the catalase content may be greater in the sediment than in the supernatant urine. If the urine has been standing for some time, however, the catalase will have left the cells, and in that case one gets the same values, whether one measures the catalase in the filtered urine or in the sediment. So, as a rule, filtration may be regarded as unnecessary.

As to the crystalline deposit, urates and oxalates are indifferent, while uric acid crystals can in certain cases produce a non-specific decomposition of the hydrogen peroxide. Phosphates seem to be indifferent, while ammonium salts and carbonates give a non-specific decomposition which may be obviated with the use of boric acid.

The volume of oxygen liberated is observed after the reaction has ceased. With small amounts of catalase the process is ended after 3 hours; with greater quantities it takes longer, but never

more than 24 hours. Consequently one may always note the results after 24 hours. The specific decomposition has then reached its maximum. If one waits longer, one sees a continued evolution of oxygen. This is corrected for by a control test in which the pyometer is filled with hydrogen peroxide but the solution of catalase is not added. The elevation in the control test will amount to about 0.1 (= 1 mm.) in 24 hours at usual room temperature and with the solution of hydrogen peroxide (Oxydol, Petri) that I have used.

The results depend on the quantity of pus and blood that are contained in the urine. Where the catalase is caused exclusively or chiefly by pus, one will, as a rule, find HPU values from 0.1 to 20, most frequently fluctuating between 1 and 10. According to catalase content, one may divide pyuria in groups.

In all of the more than 1,000 cases examined, it has been shown that excellent agreement exists between the catalase content and the pus content estimated in other ways (microscopy, Donner's test).

Clinical Results.—1. Normal urine contains only inconsiderable traces of catalase corresponding to the few epithelial cells and leucocytes which are normally found.

2. The catalase values in urine from patients suffering from pyelitis give a practical measure of the amount of cells in the urine sample.

3. By the proposed method of estimation of catalase one can divide pyurias into limited groups according to whether the catalase content is small or great, and it is easy to distinguish among (*a*) acute pyurias, where catalase is only temporarily present, (*b*) chronic pyurias, where the catalase numbers fluctuate about the same value for a longer time before they slowly approach zero, and (*c*) intermittent pyurias where the catalase numbers fluctuate widely between low and high values.

4. The method permits a reliable estimate of the effect of therapeutic measures on the condition.

Fig. 2 shows different types of pyuria.

FIG. 2.

Estimation of Catalase in Chyme.

In a great number of cases I have examined the concentration of catalase in chyme and gastric juice with the technique described above. For the sake of comparison and for practical reasons I have chosen the usual clinical test meals and fasting gastric juice.

Determination.—In all cases the filtrate from the test meal has been used for examination. Usually 0.2 cc. of filtrate has been added to the hydrogen peroxide in the pyometer, and only where the decomposition was so violent that the catalase value could not be estimated in the pyometer (HPU values over 15 to 20), fractions of 0.2, namely 0.05 or 0.02 cc., have been used, the fractional proportion being taken into consideration by the calculation of the HPU value of the filtrate. If, for instance, the value 10.2 was found with 0.05 cc. of filtrate, the HPU number in the filtrate is four times as great, or 40.8.

When the filtrate from the test meal possessed a reaction near the neutral point, a couple of boric acid crystals have been added.

Catalase in the Food.—The test meals are very poor in catalase, because this enzyme is destroyed in the preparation of the food. If the filtrate from the chyme gives catalase reaction, this must be caused by the addition of catalase from the organism, and may proceed from the cavity of the mouth, from the esophagus, or from the stomach.

Catalase from the Mouth.—This originates chiefly from the salivary corpuscles, but also in some degree from the epithelial cells of the mouth. In the examination of specimens of saliva from healthy and ill people I have found HPU numbers, which have varied between 0.8 and 14.8, an average of 5.7. In a case of stomatitis much higher values were noted. The samples of saliva must be acidulated with boric acid before the estimation.

Catalase of the Secretions of the Esophagus.—This does not seem to differ from that of the saliva.

Catalase of the Chyme.—This may originate from pus cells, red blood corpuscles, or the epithelial cells of the stomach.

Clinical Results.—1. In healthy persons the filtrate from a test meal is found free from catalase.

2. In case of hypersecretion or hyperacidity the same is true.

The value of the catalase concentration is directly dependent on the presence of free hydrocholoric acid (reaction by Boas' test). In the examination of 800 acid test meals catalase was never found in the filtrate when the test meal contained free hydrochloric acid.

3. One finds a positive catalase reaction in a number of cases of anacidity (achylia gastrica and cancer ventriculi).

4. In simple chronic gastritis (achylia gastrica) a positive catalase reaction in the filtrate has been found in a great number of cases. The HPU values are dependent on the acidity. In single cases, as well as in different ones, the rule is that the HPU values are inversely proportional to the total acidity.

5. 60 cases of gastritis of very different origin have been examined. None of these groups differs so much from the others that the supposition of a particular form of inflammation thereby is affirmed. The HPU values varied between 0 and 20. Usually the value was lower than 6.0.

6. Leucemia (lymphatica and myeloidea) does not cause a particular increase of the catalase concentration in the filtrates. In these diseases a secretion of leucocytes from the mucosa of the stomach has therefore hardly taken place.

7. Cancer cardiæ does not differ from the simple chronic gastritis in regard to the concentration of catalase in the chyme.

8. In cancer corporis ventriculi a considerable increase of the catalase concentration in the filtrate is found, even where the test meal is not mixed with blood. The catalase is supposed to proceed from the disintegration of cells. The HPU values varied between 20 and 84.

9. In cases of cancer pylori without stagnation the catalase concentrations of the filtrates were found to be the same as by the simple gastritis.

10. Where cancer pylori was followed by stagnation (lactic acid), the catalase reaction did not take place.

11. The catalase content of chyme varies inversely with the ability of the stomach to produce hydrochloric acid, and directly with the necrosis and disintegration of cells.

12. *Negative catalase reaction* in the filtrates is therefore found partly under normal circumstances, partly in hyperacidity, and partly in some cases of simple gastritis and in cancer pylori with stagnation in the stomach.

Moderate catalase reaction is found in simple gastritis, in cancer cardiæ, and in cancer pylori without stagnation (HPU values 0 to 20).

Violent catalase reaction is found only in cancer corporis ventriculi (HPU values over 20; empirically the limit is found to be 20 in the cases examined).

Estimation of Catalase in Feces.

The estimation of pus in feces is rather difficult. The microscopical examination for pus cells gives positive results only in a small number of cases, because the pus corpuscles are quickly destroyed through the action of the bacteria and the intestinal juice.

The symptoms of intestinal inflammation—pain, diarrhea, and admixture of mucus to the feces—are not quite pathognomonic. They may arise reflectorily, and they do not necessarily indicate an inflammation. It is otherwise with the proliferation of pus cells. Pus is a measure of the extent of the inflammation, and an estimation of the pus corpuscles therefore allows one to judge the extent of the inflammation. The estimation of catalase is an estimation of cells.

Determination.—The quantity of catalase is examined in an extract of the feces.

About 5 gm. of feces are weighed and mixed with three times as much half saturated boric acid solution to a homogeneous fluid. The mixture is allowed to stand 1 hour, then it is filtered; the filtration generally proceeds slowly. The catalase ought to be estimated within a half hour, as the concentration of the catalase in the filtrate diminishes on standing. For the examination one usually uses 0.2 cc. of the filtrate, but when the quantity of catalase is great, one must use smaller portions, 0.05 or 0.02 cc. In the calculation of the HPU number of the feces the used quantity of filtrate as well as the proportion 1 : 3 must be taken into consideration. The further technique is as described above. If after 24 hours with 0.02 cc. of the filtrate, the HPU number 10.2 is noted, the HPU number of the feces is $10.2 \times 10 \times 4 = 408$.

The catalase of the feces originates from the intestinal tube.

In the foregoing it was shown that the food was neary catalase-free when it left the stomach. Where this is not the case,—in achylia gastrica—the catalase is destroyed by the enzymes of pancreas, by trypsin, and erepsin. The catalase in the feces therefore originates from the pus corpuscles, the red blood corpuscles, the epithelial cells, and the bacteria of the intestines. The estimations show that bacteria and epithelial cells are of secondary consequence. The main part of the catalase comes from the red and white blood corpuscles. Therefore with every catalase reaction a blood reaction should be performed to ascertain how much blood there is in the feces. Not all blood is of importance for the catalase reaction in feces. Blood from the food and blood which comes from the mouth, esophagus, and corpus ventriculi are of no importance for the estimation of catalase in feces, as the catalase in the blood is destroyed in the stomach; on the other hand, bleeding ulcers near the pylorus and in the intestine will increase the concentration of catalase in the feces.

Clinical Results.—1. In normal feces the average of the HPU numbers is 1.5. The lowest value found is 0.2 and the highest 3.5 in normal cases. The numbers vary most often between 0.5 and 2.5. The softer the feces, the lower is the HPU number (Fig. 3, No.1). In pathological cases the values may reach 1,000. In the clinical examination there is a great interval between the physiological and the pathological values.

2. There are some cases of gastritis (achylia gastrica) in which the HPU members lie within the same limits as the normal.

3. In bleeding gastric ulcer the blood in most cases causes an increased quantity of catalase in the feces, when the ulcer is situated at the pylorus or in the duodenum, or when a gastroenterostomia has been made. Normal numbers (0.5 to 3.5) are found when the ulcer is in the corpus ventriculi near the cardia.

4. During the treatment with milk for gastric ulcer, as well as in all other cases where the food consists of milk for a long period, a reaction (enteritis) from the intestine will occur sooner or later.

5. In bleeding gastric ulcer one may often see an increase in the HPU numbers of the feces, at first as a consequence of the hemorrhage, later as a consequence of the treatment with milk (Fig. 3, No. 2).

6. In uncomplicated constipation (intestinal stasis) the catalase concentration of the feces is decreased in the filtrate, because the catalase has been destroyed during the stay in the intestinal canal (HPU numbers 0.2 to 1.0, Fig. 3, No. 3).

7. In cases of achylia with constipation the same low numbers are found.

8. Nervous diarrhea has low HPU numbers. So it must be interpreted as an abnormal peristalis and not as an inflammation (Fig. 3, No. 4).

9. In Graves' disease with diarrhea we find both increased peristalis and a transudation of a secretion containing catalase.

10. Diseases of the intestines with diarrhea may be divided into two groups: (*a*) the diseases where the HPU numbers are not increased (nervous diarrhea, diarrhea in Graves' disease, flesh poisoning, cases of mucus and membranaceous colitis, diarrhea in influenza, and by a few cases of achylia; (*b*) the diseases where the HPU numbers are increased (infectious forms).

11. In intestinal fermentation acid products are found in the intestinal canal. These destroy the catalase. Consequently one finds low HPU numbers, where this disease is not connected with a secondary inflammation (Fig. 3, No. 5).

12. In several cases hemorrhages from the intestinal canal have caused high HPU numbers in the feces. Here one may distinguish between a *vascular* type (Fig. 3, No. 6), where the increase is great, but of short duration and with intercalated low or normal numbers, and a *parenchymatous* type (Fig. 3, No. 7), where the elevations are more or less high but where normal values are lacking.

13. Bleeding hemorrhoidal tumors give HPU numbers of the vascular types, cancer coli and cancer recti, of parenchymatous type.

14. The diseases of the intestine which are caused by infections have high HPU numbers. This is the case in the usual acute, infectious enteritis (Fig. 3, No. 8) and in the usual chronic colitis and enterocolitis (Fig. 3, No. 9).

15. Colitis gravis (colitis chronica suppurativa) is recognized by the microscopical finding of several pus cells in feces. The HPU numbers in these cases are very high (Fig. 3, No. 10.). The same is found in proctitis suppurativa. The fluctuations of the HPU numbers may give an idea of the prognosis.

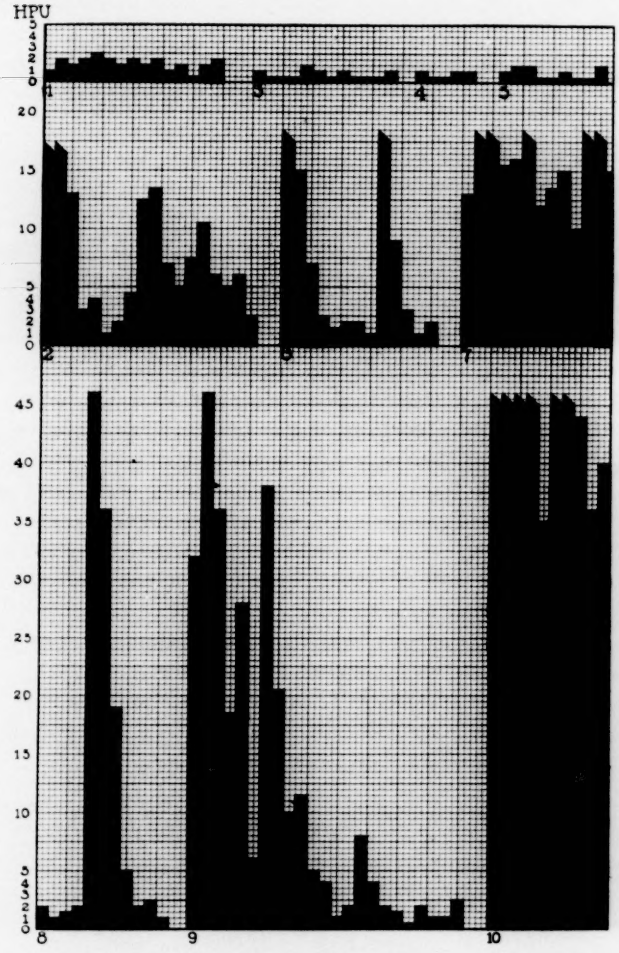

FIG. 3. 1. Normal. 2. Gastric ulcer (observe the two elevations of the curve). 3. Intestinal stasis. 4. Nervous diarrhea. 5. Intestinal fermentation. 6. Vascular hemorrhage. 7. Parenchymatous hemorrhage. 8. Enteritis acuta. 9. Colitis chronica. 10. Colitis suppurativa chronica.

16. Dysentery, typhoid fever, paradysentery, and paratyphoid fever present very high HPU numbers, as long as inflammation is present in the intestine.

17. Tubercular enteritis presents the same picture as colitis chronica and colitis gravis. The HPU numbers fall during the treatment.

18. Enteritis, which arises in connection with mercurial or arsenical treatment, shows the same type as the infectious enteritis.

19. In a large number of cases of constipation the patients examined have shown symptoms of enteritis (high HPU numbers). These are caused by irritation of the intestinal wall (colitis stercoralis).

20. In a great number of cases of achylia one finds periodically symptoms of enteritis. These periods arrive at various long intervals and are generally of short duration. These enteritides depend on the predispositon of the patients for infection, as the disinfecting HCl of the stomach is lacking.

21. The feces from patients with cancer ventriculi do not differ from those of patients with achylia in regard to the catalase concentration.

22. In most cases of intestinal fermentation one finds an enteritis which may be cured by a suitable treatment.

23. Parenteral diseases which cause abdominal pain do not increase the quantity of catalase in the feces.

24. An increase of the HPU numbers in the feces is a clinical symptom of an inflammation in the intestine and is therefore not the same as an admixture of mucus to the feces.

Modified Technique for Practical Use.

A particle of feces as large as a pea is smeared out on a clean surface (glass or porcelain). The smear is allowed to dry a little at room temperature. Then a few drops of 3 per cent hydrogen peroxide are added.

In constipation, intestinal fermentation, and non-infectious diarrhea the liberation of oxygen is minimal.

With normal feces but slight oxygen liberation takes place. In infectious diseases of the intestine or on addition of fresh blood a violent evolution of oxygen occurs. It is very violent if an abundant quantity of pus is present. In this case it is advisable to test for blood with the benzidine reaction.

CAN "HOME GROWN RATIONS" SUPPLY PROTEINS OF ADEQUATE QUALITY AND QUANTITY FOR HIGH MILK PRODUCTION?*

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In modern dairy farming clover or alfalfa has become recognized as an almost indispensable roughage. The farmer has been taught that these materials are high in protein, in consequence of which they can supplement his more starchy grains and low protein silage. Therefore, the "home grown ration," consisting of the cereal grains, silage, and clover or alfalfa hay would become, in the minds of some farmers and teachers, sufficiently adequate in protein content for high milk production.

With the newer view-point of protein chemistry emphasizing the fact that the value of a protein mixture for growth or milk production will depend upon its qualitative and quantitative make-up and not merely on the quantity of the proteins ingested, it is impossible to state whether the "home grown ration" would furnish a protein supply of proper quality for high milk production without drawing on the protein tissue reserves of the animal.

The question raised can be settled only by metabolism investigations. In the present state of our knowledge it is just as probable as not that a ration made of clover hay, corn silage, and corn-meal would furnish an excellent and adequate protein mixture. The time is past for set nutritive ratios. A protein intake that will keep a cow in nitrogen equilibrium and maintain her flow of milk is all that is required and the quantity of proteins necessary to accomplish this will depend on the source of the proteins and on the quality of the amino-acids they can furnish.

In milk production we have undoubtedly followed in the past

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

a safe procedure by using a narrow nutritive ratio, which implies a liberal protein intake, for all the evidence accumulated indicates that the common sources of proteins used in feeding dairy cows,—and they have always been plant sources—will not yield a protein mixture of the highest possible production value. Our experience with a "home grown ration," the results of which are here reported, is in harmony with this view. A protein mixture derived from clover hay, corn silage, and corn grain, oat grain, barley grain, or a mixture of the three grains will be a protein mixture of comparatively low efficiency for maintenance and milk production. It will suffice for cows of large consumption capacity and low mammary gland activity, in other words, for poor cows. But where a cow comes into lactation with a large milk flow her consumption capacity will be inadequate to furnish from such a ration sufficient protein for a positive nitrogen balance or equilibrium and the maintenance of a high milk yield. The result is a partial withdrawal of tissue nitrogen for milk production with an accompanying rapid decline in milk flow.

Undoubtedly the fact that generally in dairy practice the proteins used are of low production value and that the plane of protein intake often fed dairy cows is lower than it should be, is partly responsible for the rapid decline in milk production during the progress of lactation. Probably more cows than imagined are in negative nitrogen balance during the early period of lactation and under such conditions rapidly decline in milk flow to offset the losses sustained by autolyzing tissue.

It is undoubtedly true that some cows of strong milking tendencies and with a considerable reserve of muscular tissue will maintain for a long time a constant level of milk protein production even when in a negative nitrogen balance, but more often the tendency will be toward a condition of equilibrium.[1] That is exactly what happened in the experiments to be detailed below.

The experiments reported here involve only clover hay, corn silage, and the cereal grains. While the high protein legume seeds, such as peas and beans, are "home produced" yet their relatively low yield per acre and high market value for human food have not made them common adjuncts to ration construction on dairy farms.

[1] See records of Animals 1 and 2, Charts 1 and 2, *J. Biol. Chem.*, 1918, xxxv, 367.

EXPERIMENTAL.

The plan followed was to use a ration of corn silage and clover hay (medium red) to which ground oats were added in the first period of 4 weeks; ground barley in the second period of 4 weeks; ground corn in the third period of 4 weeks; and a mixture of approximately an equal part of the three grains in the fourth period of 4 weeks. Provision for a constant energy supply in the different rations was made through the use of corn-starch. The proportion of grains and starch fed to Cows 1, 2, and 4 was in conformity to a rule of animal husbandry teachers, namely, 1 pound of grain to 3 pounds of milk, while in the case of Cows 3 and 5 a departure from this rule was made. In the latter cases a larger proportion of grain was used.

Originally our plan was to use but three animals, carrying each one through four succeeding periods of 4 weeks each. This, however, could not be accomplished. Cows 1 and 3 were fairly large producers, producing 34 to 37 pounds of milk daily at the time the experiment was initiated. Although these cows were given all the ration they would consume they were in a negative nitrogen balance from the beginning of the experiment for from 5 to 6 weeks and gradually decreased their milk flow to a point where the ration consumed maintained a state of nitrogen equilibrium; this point was reached before the termination of the second feeding period. To continue these individuals during the next 4 weeks feeding period with the corn grain ration would not have answered the question for which we sought an answer, namely, could our protein mixture derived from corn grain, clover hay, and corn silage maintain a high milk production and nitrogen equilibrium? Consequently two other animals, Cows 4 and 5, were substituted at this point and continued through the corn and mixed grain periods.

Two of the animals used were pure bred Guernseys, one a grade Jersey, and two pure bred Holsteins. They were fed twice daily, the excreta were quantitatively collected and analyzed daily, while the milk was a 7 day composite sample. The composition of the rations fed is shown in Table I. A nutritive ratio of approximately 1:8.8 was obtained, with a total protein intake varying from 2.1 pounds per day for the smaller grade Jersey

TABLE I.

Composition of Rations.

Oat grain ration, fed daily.

	Animal 1.			Animal 2.			Animal 3.		
	Weight.	Total N.	Therms.	Weight.	Total N.	Therms.	Weight.	Total N	Therms.
	lbs.	*gm.*		*lbs.*	*gm.*		*lbs.*	*gm.*	
Silage.....	25.5	37.1	4.22	25.5	37.1	4.22	25.2	44.0	4.15
Clover Hay..	10.2	83.3	3.53	10.2	83.3	3.53	7.2	56.7	2.49
Grain.....	8.1	56.7	5.35	8.1	56.7	5.35	9.9	76.4	6.55
Starch.....	2.1	—	2.10	2.1	—	2.10	3.6	—	3.60
Total.....	45.9	177.1	15.2	45.9	177.1	15.2	45.9	177.1	16.79

Barley grain ration, fed daily.

	Animal 1.			Animal 2.			Animal 3.		
Silage.....	23.7	35.9	3.95	25.5	37.1	4.2	23.7	35.9	3.95
Clover hay...	9.5	74.9	3.29	10.2	83.3	3.65	6.7	54.4	2.32
Grain.....	6.2	54.2	5.16	6.7	56.7	5.31	7.6	64.5	6.03
Starch.....	1.9	—	1.90	2.0	—	2.00	3.4	—	3.40
Total.....	41.3	165.0	14.3	44.4	177.1	15.1	41.4	154.8	15.7

Corn grain ration, fed daily.

	Animal 4.			Animal 2.			Animal 5.		
Silage.....	26.6	43.1	4.4	25.5	37.1	4.3	28.9	45.4	4.8
Clover hay...	10.6	83.5	3.6	10.2	83.3	3.5	8.2	64.4	2.8
Grain.....	8.7	58.7	8.1	8.3	56.7	7.3	11.7	80.5	10.2
Starch.....	—	—	—	—	—	—	1.2	—	1.2
Total.....	45.9	185.3	16.1	44.0	177.1	15.1	50.0	190.3	19.0

Mixed grain ration, fed daily.

	Animal 4.			Animal 2.			Animal 5.		
Silage.....	26.6	43.1	4.4	25.5	37.1	4.3	28.9	45.4	4.8
Clover hay...	10.6	83.5	3.6	10.2	83.3	3.5	8.2	64.4	2.8
Oats.....	2.7			2.6			3.6		
Barley.....	2.2	55.0	6.2	2.1	53.9	5.8	3.0	75.4	9.3
Corn.....	2.9			2.8			3.9		
Starch.....	1.4	—	1.4	1.4	—	1.4	2.5	—	2.5
Total.....	46.4	181.6	15.6	44.6	174.3	15.0	50.1	185.2	18.4

(No. 3) to 2.6 pounds for the larger Holstein animals (Nos. 4 and 5). In some instances not all the ration allowed was consumed. The nitrogen content of these residues was deducted from the total nitrogen fed. In Tables II, III, and IV are given the records

TABLE II.

Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Oat grain period, Animal 1.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 3– 9	1,239.7	544 3	239.3	516.9	− 60.8	227.8
" 10–16	1,239.7	547.9	211.9	510.2	− 30.3	230.6
" 17–23	1,117.1	538.3	228.3	483.0	−132.5	226.1
" 24–30	1,013.6	488.0	183.0	439.4	− 96.8	208.8
Barley grain period, Animal 1.						
Dec. 31–Jan. 6	1,150.0	518.6	209.8	439.1	− 17.5	204.6
Jan. 7–13	1,155.0	527.5	197.9	404.5	+ 25.1	180.7
" 14–20	1,155.0	563.6	205.3	390.5	− 4.4	187.8
" 21–27	1,155.0	539.7	211.8	388.9	+ 14.6	171.8
Corn grain period, Animal 4.						
Jan. 28–Feb. 3	1,321.4	628.4	391.0	429.8	−127.8	210.5
Feb. 4–10	1,297.1	631.7	360.1	408.7	−103.4	191.3
" 11–17	1,297.1	617.4	366.4	369.4	− 46.1	178.0
" 18–24	1,297.1	668.7	402.4	358.7	−132.7	158.6
Mixed grain period, Animal 4.						
Feb. 25–Mar. 3	1,271.2	613.2	341.8	336.2	− 20.0	157.6
Mar. 4–10	1,271.2	608.2	310.9	342.3	+ 9.8	136.4
" 11–17	1,271.2	581.7	302.5	339.1	+ 76.9	133.5
" 18–25	1,271.2	600.2	310.5	332.4	+ 28.1	132.9

of nitrogen balance. These figures represent the intake and outgo for a 7 day period. Charts 1 to 5 graphically illustrate the nitrogen metabolism.

A study of the data shows that Cows 1 and 3 were in a decidedly negative nitrogen balance on the oat grain ration and passed to

a status of nitrogen equilibrium during the feeding of the barley ration. This is not to be interpreted as indicating a better protein mixture with the barley grain ration, but as due to a decreased production of milk nitrogen during the progress of the experi-

TABLE III.

Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Oat grain period, Animal 2.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 3–9	1,239.7	546.1	297.6	405.9	− 9.9	167.6
" 10–16	1,239.7	536.7	307.8	409.8	−14.6	169.7
" 17–23	1,239.7	564.7	289.6	395.7	−10.3	167.4
" 24–30	1,239.7	541.3	300.2	376.9	+19.3	165.2
Barley grain period, Animal 2.						
Dec. 31–Jan. 6	1,239.7	580.2	264.4	356.0	+39.7	154.7
Jan. 7–13	1,239.7	525.4	264.4	348.4	+99.5	149.5
" 14–20	1,239.7	550.0	286.5	317.0	+76.2	147.9
" 21–27	1,239.7	534.7	290.1	324.8	+90.1	139.0
Corn grain period, Animal 2.						
Jan. 28–Feb. 3	1,239.7	555.4	297.7	328.4	+58.2	141.0
" 4–10	1,239.7	593.4	325.7	362.8	−52.2	150.8
" 11–17	1,239.7	575.8	304.1	367.4	− 7.6	147.4
" 18–24	1,239.7	565.2	334.9	363.1	−23.5	140.0
Mixed grain period, Animal 2.						
Feb. 25–Mar. 3	1,220.1	566.8	332.5	354.7	−33.9	138.6
" 4–10	1,220.1	577.0	301.3	337.5	+ 4.3	139.9
" 11–17	1,220.1	602.5	303.0	342.0	−27.4	141.3
" 18–25	1,220.1	580.5	305.8	335.5	− 1.7	140.7

ment, with less drain on the fragments of tissue autolysis. The initial daily milk production of these two animals was well over 30 pounds and from the data it is clearly evident that such a ration will contain too poor a protein mixture for the production of so large a quantity of milk proteins without a considerable

drain on the animal's tissues. Animal 1 during the 8 weeks of feeding the oat and barley grain rations decreased the amount of nitrogen secreted in the milk per week by over 100 gm.; Animal 3 likewise decreased the milk nitrogen produced in a week by

TABLE IV.

Record of Nitrogen Balance, Milk Production, Etc.

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Oat grain period, Animal 3.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 3–9	1,200.0	497.2	206.0	542.7	− 45.9	247.9
" 10–16	1,164.7	544.9	211.8	514.4	−106.4	228.6
" 17–23	1,039.2	496.4	168.2	457.1	− 82.5	229.4
" 24–30	984.2	435.5	168.1	435.9	− 55.3	205.0
Barley grain period, Animal 3.						
Dec. 31–Jan. 6	965.2	453.0	157.8	398.8	− 44.4	195.4
Jan. 7–13	999.6	468.7	135.9	402.9	− 7.9	184.8
" 14–20	1,002.0	470.0	142.1	377.0	+ 12.9	184.4
" 21–27	978.0	446.9	139.7	380.3	+ 12.1	168.5
Corn grain ration, Animal 5.						
Jan. 28–Feb. 3	1,296.4	685.4	266.0	420.6	− 75.6	198.9
Feb. 4–10	1,332.1	723.8	261.8	405.6	− 59.1	184.8
" 11–17	1,332.1	725.7	265.7	386.3	− 45.7	187.4
" 18–24	1,332.1	708.8	294.5	375.2	− 46.4	166.6
Mixed grain period, Animal 5.						
Feb. 25–Mar. 3	1,296.4	662.8	260.9	371.1	+ 1.6	165.7
Mar. 4–10	1,296.4	708.8	203.6	371.2	+ 7.2	165.7
" 11–17	1,296.4	714.1	216.6	370.9	− 5.2	163.5
" 18–25	1,296.4	710.1	218.8	365.4	+ 2.1	163.5

150 gm. The volume of milk produced by No. 1 on December 3, when the experiment was started, was 33.8 pounds, but 8 weeks later, or on February 3, it was but 23.7 pounds. No. 3 produced on December 3, 36.9 pounds of milk, but on February 3, or 8 weeks later, she produced but 25.9 pounds.

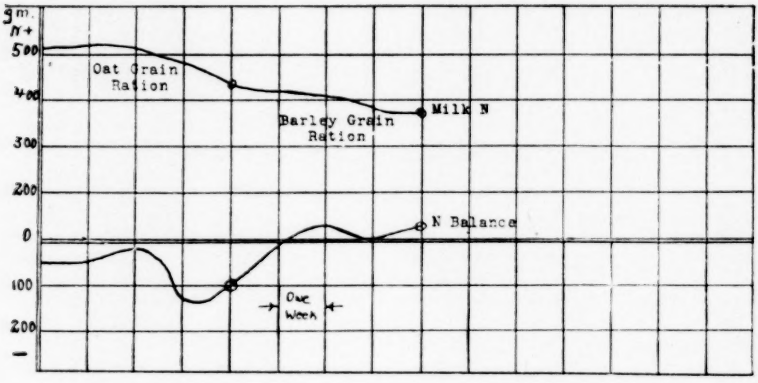

CHART 1. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

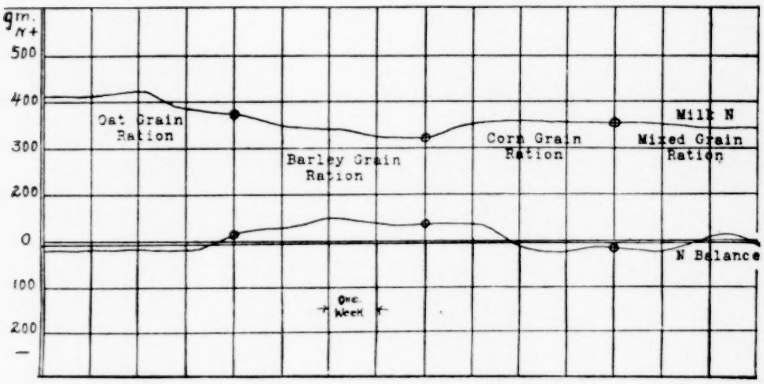

CHART 2. Showing nitrogen balances and milk nitrogen produced. This animal had a large consumption capacity and practically maintained her nitrogen equilibrium from the beginning of the experiment. The result was that over a period of 16 weeks the gradual decrease in weekly milk nitrogen elaborated was not large.

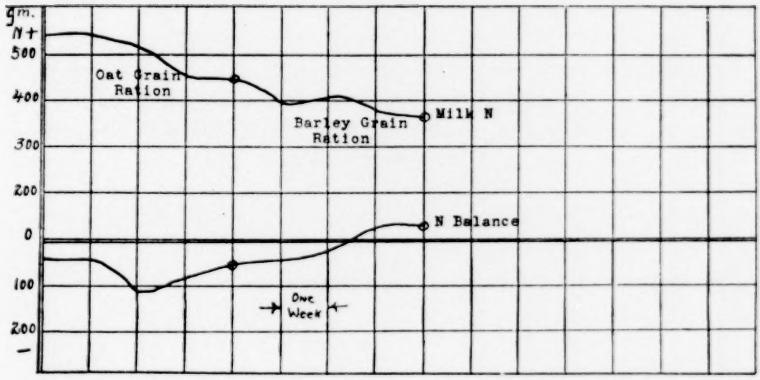

CHART 3. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

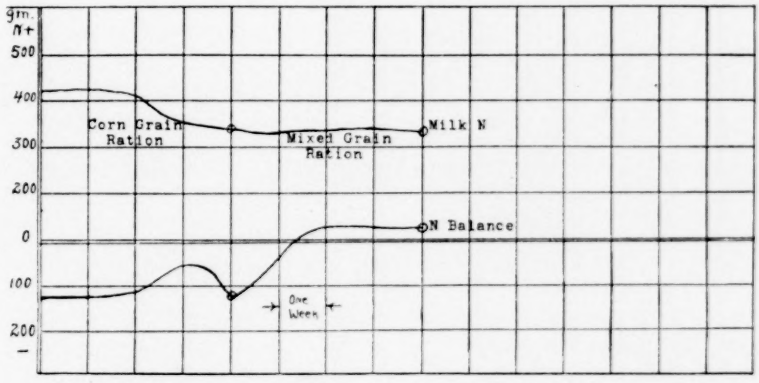

CHART 4. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

Animal 2 had a large capacity for feed, but a much lower milk-producing capacity than the other two animals, Nos. 1 and 3. The result was that she was able to maintain herself in nitrogen equilibrium with the amount and quality of the nitrogen provided by a ration of corn silage, clover hay, and the cereal grain, or a cereal grain mixture. She was continued under observation for the entire 16 weeks and during that time decreased the milk nitrogen produced per week by but 60 to 70 gm. The volume of milk also was remarkably maintained, starting with a production on December 3 of 23.7 pounds and completing the record

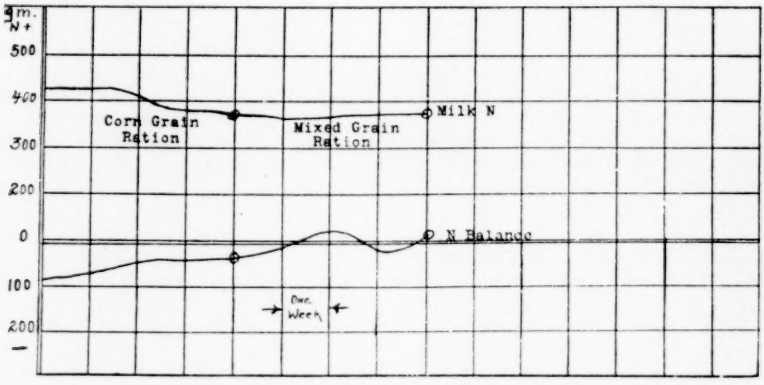

CHART 5. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

on March 17 with 20.1 pounds. This animal's record is an excellent demonstration of the fact that only moderate milk production is possible from the common "home grown" products used in these experiments.

In Table V sufficient data are presented to support the conclusion that the essential change incident to a protein intake insufficient in quantity or quality for nitrogen equilibrium in a milking animal is a rapid decrease in milk flow and not a change in the percentage composition of the milk produced.

Animal 4 (see Table II) starting with all she could consume of the corn grain ration and with a fairly high milk production

(33.3 pounds of milk per day) was immediately plunged into a negative nitrogen balance. The sequel to this condition was a rapid decrease in milk flow. Apparently the corn grain ration gave no better protein mixture than did the oat grain or barley grain ration. This animal reached nitrogen equilibrium in the mixed grain ration (oat-barley-corn mixture), but only after a decrease of 90 gm. per week in milk nitrogen elaboration and a

TABLE V.

Decrease in Milk Volume with Cows in Negative Nitrogen Balance, but Maintenance of Percentage Composition of Milk.

	Animal 1.		Animal 4.	
	Dec. 16.	Jan. 13.	Feb. 10.	Mar. 10.
Total solids, *per cent*	13.57	13.52	10.96	11.90
Fat, *per cent*	4.60	4.45	2.80	3.50
Nitrogen, *per cent*	0.49	0.47	0.41	0.48
Milk daily, *lbs.*	32.7	26.8	32.9	19.6
	Animal 2.*			
Total solids, *per cent*	14.27	14.26	13.88	14.17
Fat, *per cent*	4.70	4.60	4.70	5.10
Nitrogen, *per cent*	0.53	0.51	0.55	0.53
Milk daily, *lbs*	24.3	21.60	22.2	21.2
	Animal 3.		Animal 5.	
Total solids, *per cent*	13.07	12.73	12.07	12.58
Fat, *per cent*	4.10	3.50	3.10	3.60
Nitrogen, *per cent*	0.46	0.47	0.45	0.49
Milk daily, *lbs*	32.6	26.0	31.3	23.0

* Cow 2 maintained volume of milk, but was not in negative nitrogen balance.

shrinkage in daily milk volume from 33.3 pounds per day to 19.5 pounds per day.

The behavior of Animal 5 was an exact duplication of the records of the other high producing animals with which we experimented. There was a decided negative nitrogen balance on the corn grain-corn silage-clover hay ration, with a progressive decrease in flow of milk until nitrogen equilibrium was reached. In the case of this individual there was a decrease in daily milk flow from 31.3

pounds at the beginning of the experiment to 23.6 pounds at the time that nitrogen equilibrium was reached and the weekly amount of nitrogen secreted in the milk was reduced by 50 gm. in 4 weeks.

Our data on the value of the proteins of the mixed grain ration are not so conclusive as those on the single grain mixtures. All the animals had practically reached nitrogen equilibrium when they were changed to the mixed grain ration. From available evidence on the value of a mixture of cereal grain proteins for growth[2,3] it is altogether probable that such a mixture of grains as used here would furnish no better quality of proteins than the single grain itself. The elaboration of milk proteins is to be looked upon as a phenomenon analagous to growth. While the distribution of amino-acids in the milk proteins is different from what it is in new tissue formed in growth, yet their origin in either case is the food supply, since probably no distinctive capacity for amino-acid synthesis is inherent in the mammary gland.[4] Further, had the amino-acid content of the mixed grain ration been of a quality superior to that of any of the single grain mixtures used, there would have been a larger storage of nitrogen following the periods of negative nitrogen balance experienced during the corn grain period. This, however, was not the case (see records of Animals 4 and 5, Tables II and IV).

The live weight of our animals gave no indication in the period of observation as to the status of their nitrogen equilibrium. Two of them, Nos. 1 and 3, lost considerable weight under conditions of a negative nitrogen balance; No. 2 which was in nitrogen equilibrium during the entire 16 weeks neither gained nor lost appreciably in weight. Nos. 4 and 5, although in negative nitrogen balance 4 to 5 weeks showed no decrease in live weight,—in fact they actually increased in weight. Records of live weight are given in Table VI.

The animals in negative nitrogen balance decreased their milk flow in the course of 5 to 6 weeks by 8 to 10 pounds. This amount of milk would have a market value of at least 50 cents. For 7 cents per day 2 pounds of any of the common plant protein con-

[2] McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.

[3] Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, 1919, xxxviii, 267.

[4] Hart, E. B., Nelson, V. E., and Pitz, W., *J. Biol. Chem.*, 1918, xxxvi, 291.

centrates could have been added to the ration, and thereby raised the plane of protein intake, without the animals experiencing any such rapid shrinkage in milk flow as took place. From what we know of the physiology of milk secretion and the relation of protein to that function it would appear to be unprofitable not to supply protein in quantities sufficient to maintain nitrogen equilibrium in a milking animal.

TABLE VI.

Records of Live Weights of Animals.

Animal No.	December 2.	January 20.	March 17.
	lbs.	*lbs.*	*lbs.*
1	1,024	935	
2	1,088		1,109
3	885	762	
4		1,356	1,395
5		1,135	1,200

SUMMARY.

1. In this paper data are presented which show that it is not possible to furnish dairy cows of *high milk-producing capacity* with a protein level of sufficient magnitude or quality *to maintain that capacity* from a clover-corn silage-cereal grain mixture, the latter being corn, barley, or oats alone or a mixture of the three.

2. A cow with low mammary capacity but with large food consumption can be kept in nitrogen equilibrium by such rations. One of our animals gave 22.24 pounds of milk daily for 16 weeks and maintained nitrogen equilibrium on such a ration.

3. In the present state of our knowledge the only safe procedure for the maintenance of high milk production is through the use of a high plane of protein intake which, although of low relative efficiency, should be drawn from the plant protein concentrates rather than from those of animal origin.

Further studies will be made of the possibilities of other "home grown" protein combinations. It is entirely possible that highly efficient protein mixtures used at comparatively low levels of intake may yet be found among the common plant sources.

THE USE OF TURPENTINE RESIN IN TURPENTINE AS A FOAM BREAKER.

By E. C. KENDALL.

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(Received for publication, May 23, 1919.)

Many substances have been suggested for breaking the foam which is formed by the rapid aeration of blood and other fluids. Among the best of these agents are caprylic and amyl alcohol. If only an occasional determination is made, caprylic alcohol or amyl alcohol is entirely satisfactory and the cost is negligible, but if a large amount of work is carried on for a long time the cost of material becomes of importance.

During the past year attempts have been made in our laboratory to find a cheaper substitute for amyl alcohol. Among other substitutes, turpentine was found to have distinct foam-breaking properties. As the sample tried was not quite neutral it was decided to distill it and it was then found that the distilled turpentine had lost practically all its foam-breaking properties. The real foam-breaking agent appeared to be some impurity which was contained in crude turpentine and which could be removed by distillation. The most probable impurity was resin, and we found that on adding resin to turpentine its foam-breaking power was greater than that of amyl alcohol. We now use from 1 to 2 cc. of a 20 per cent solution of resin dissolved in turpentine. This is made by breaking the resin into small pieces and dissolving it in the turpentine on a hot water bath. During the last six months we have encountered no blood which could not be aerated at the most rapid rate with this foam-breaking agent. If the solution of resin in turpentine is not neutral to alizarin, either 0.1 N sodium hydroxide or sulfuric acid is added until the solution is exactly neutral. Resin dissolved in turpentine is an efficient foam-breaker, is very easily prepared, and is much cheaper than caprylic or amyl alcohol.

THE HYDROGEN ION CONCENTRATION OF FOODS.

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(*From the Physiological Laboratory, University of Minnesota Medical School, Minneapolis.*)

(Received for publication, May 29, 1919.)

Owing to the fact that the vitamines of foods are more perishable in alkaline than in acid media, it was thought worth while to increase our knowledge of the hydrogen ion concentration of foods. Such determinations may also be useful in connection with a study of the hydrogen ion concentration of the contents of the intestinal tract.

Foà[1] states that the pH of ripe grape juice is 4.52, of the juice of a nearly ripe pear is 4.24, and of the milk of *Ficus elastica* is 5.7. Clark and Lubs[2] give a number of determinations as follows:

Substance.	Raw.	Autoclaved.
	pH	*pH*
Whey	1.64–2.56	
Vinegar	2.36–3.21	
Silage juice	3.70–3.91	
Apple "	3.76–5.65	3.8
Prune "	4.12–9.44	4.3
Beer wort	4.91–8.55	
Carrot juice	5.21–9.27	5.2
Cucumber "	5.08	5.1
Apple "	5.02	
String bean juice	5.23–8.63	5.2
Banana juice	4.62	4.6
Potato "	6.06–9.44	6.1
Sweet potato juice	5.80–8.73	
Maple syrup	6.75–6.8	
Beet juice	6.07–8.75	6.1

[1] Foà, C., *Arch. Fisiol.*, 1906, iii, 390.

[2] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

These determinations were made with the hydrogen electrode but the juices were used to standardize colorimetric methods and were apparently kept for long periods and became more alkaline, and the more acid reaction probably represents the fresh juice. They quote the following values from the literature:

	pH
Muscle juice	6.8
Pancreas extract	5.6
Milk	6.6–7.6
Flour extract	6.0–6.5
Beer	3.9–4.7
Wine	2.8–3.8
Lime juice	1.7
Lemon "	2.2
Cherry "	2.5
Grapefruit "	3.0–3.3
Orange "	3.1–4.1
Rhubarb "	3.1
Strawberry "	3.4
Pineapple "	3.4–4.1
Tomato "	4.2
Plant cell sap	5.3–5.8

Our own determinations were begun as an attempt to find the cause of the deterioration of the antiscorbutic substance between the time of its formation in the sprouting of barley and through the process of making malt extracts in various ways. We found that the acidity of press juice or extracts of steeped barley or sprouting barley or any of the products investigated was as acid as pH = 5.5 and was not made less acid by exposure to air, filtration through fullers' earth, boiling, or evaporation at low pressure while a stream of air passed through it. This acidity is somewhat lower than orange juice and much lower than lemon juice. It might therefore be advantageous to add acid to preserve the antiscorbutic substance. It would require too much space to give all the determinations with the many variations in mode of treatment of the product, but the following example illustrates the character of the work. Barley of 95 per cent germination capacity was malted by the drum method until the acrospire was as long as the grain, the green malt was crushed between steel rollers, and a little water was added to make a pasty mass and pressed in a Buchner press without the addition of any other substance. The pH of the press juice was 5.4 and of the same after boiling 5.45.

We extended our observations to foods in general and made a long series of determinations with the result that they were all on the acid side of neutrality no matter what the condition of freshness, mode of storage, stage in the preparation (cooking), or dilution with water (extract, soup, or pot-liquor). We had supposed that meats, at least, might turn alkaline on cooking, due to the loss of CO_2 in the bicarbonates contained in them, but apparently enough lactic acid is developed in meat to prevent this change. For example, a piece of lean rabbit was boiled until the muscle fibers began to loosen and the press juice pH was 6.25 whereas the pH of the pot-liquor or water in which the meat was boiled was 5.89. We did not succeed in getting enough press juice of raw meat without the addition of water, as the clotting of the muscle proteins causes the water to be held rather firmly, but the following example may illustrate the work we did do. A rabbit was killed and the leg muscles placed in a mixture of ice and salt until frozen. The frozen muscles were quickly sliced and placed between steel plates and suddenly subjected to a pressure of 5,000 pounds per square inch to press out the muscle plasma. This muscle plasma was mixed with a little water and placed in a canvas bag in a Buchner press, and the press juice run into the electrode, showing pH = 6.

We made comparisons of the pH of vegetables pressed fresh, or first cooked and then pressed and found slight differences in the pH. In order to determine whether this was due to the volatility of some acid or base we pressed the juice from the raw food and determined the pH, then boiled the juice and then determined the pH. The differences obtained were very slight and might be due to changes in acid-binding power of proteins on coagulation.

Table I shows the pH of fruits and vegetables in our last series of determinations. The juice was pressed out of the raw food and the pH determined, then the juice was run into a silica dish, an

TABLE I.

Substance.	Raw.	After boiling.
	pH	*pH*
Young carrot juice	5.85	5.80
Potato juice	5.57	
Cabbage "	5.90	5.78
Orange "	3.55	3.55
Lemon "	2.32	2.30

equal volume of distilled water added, then boiled, and the original volume restored by the addition of distilled water or further boiling, as required.

In general it may be said that the juice became slightly more acid on boiling. On the contrary, if the food was boiled before pressing it might be slightly less acid, thus raw potato gave pH = 5.57 and boiled potato 5.79, raw cabbage 5.90 and boiled cabbage 6.50.

As the technique of hydrogen ion determinations of such material has been admirably described by Clark[3] it is not necessary to go into details, but we do not believe all the precautions taken by Clark are necessary. We found it necessary to immerse the platinized part of the electrode completely but not to avoid foam, and after thorough shaking the potential was maintained constant for a long period after the shaking ceased. If the solution to be tested is sufficiently viscous to support a layer of the solution on the platinized electrode (as in the case of blood plasma), it is not necessary to take the reading with the electrode totally immersed. But in the case of most plant juices the film drains off the electrode and at the same time the potential gradually changes and continues to do so for hours and it is therefore impossible to decide the correct reading. We found that palladium-coated electrodes deteriorated so rapidly that they were discarded and platinized gold electrodes used exclusively. From other work, however, we believe iridium would be superior to platinum.

Certain unavoidable difficulties enter into the determination of raw juices that prevent absolute uniformity of results. The juices contain enzymes and, if not absolutely sterile, a variable quantity of adventitious enzymes is developed. This enzyme action may be readily observed in plant juices, due to the presence of oxidases that act on chromogens such as tyrosine. We did not make a complete study of changes in acidity due to autolysis or bacterial action, but observed isolated instances of such changes. For example, freshly pressed carrot juice gave pH = 5.85 whereas a portion of the same juice exposed to air 20 minutes before running into the electrode gave pH = 5.73. All our observations showed increase in acidity on standing, but we interpret the tables of Clark and Lubs as indicating that the juices might become alkaline, possibly due to alkaline fermentation such as occurs in urine.

[3] Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.

FACTORS INFLUENCING THE HYDROGEN ION CONCENTRATION OF THE ILEUM.

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The senior author determined the pH of duodenal contents from two adults (human) collected by Dr. Schneider in April, 1915, and found one to be 7.61 and the other to be 1.5, or an average of 4.55, whereas the average of fifteen samples from infants was 4.9. Long and Fenger[1] obtained an average of pH = 5.72 for adult human duodenal contents (thirteen samples). McClendon, Shedlov, and Thomson[2] obtained an average of pH = 6.2 for the contents of the ileum of seven pups and McClendon, Shedlov, and Karpman[3] obtained an average of pH = 6.03 in eight determinations of the ileum of four adult dogs. The discrepancy between the results on human and dog intestine may be due to the fact, as shown below, that the intestine may become less acid on the way down and the human samples could be taken from the duodenum only.

Torrey[4] has shown that lactose and dextrin when added to a basal diet for dogs cause replacement of *Bacillus coli* by *Bacillus acidophilus* and, although the above results may not show a difference in the pH of the lactose-eating puppy and adult dog, the human data indicate such a difference and we wished to test this question more thoroughly. Adult cats were fed as much lactose in milk as they would consume. The average of five determinations on the pH of the ileum was 6.01 as compared with 6.38 for six

[1] Long, J. H., and Fenger, F., *J. Am. Chem. Soc.*, 1917, xxxxix, 1278.

[2] McClendon, J. F., Shedlov, A., and Thomson, W., *J. Biol. Chem.*, 1917, xxxi, 269.

[3] McClendon, J. F., Shedlov, A., and Karpman, B., *J. Biol. Chem.*, 1918, xxxiv, 1.

[4] Torrey, J. C., *J. Med. Research*, 1919, xxxix, 415.

determinations on cats fed waste food from the table. One determination on a cat to which 200 gm. of cane sugar were administered by means of the stomach tube gave pH = 6. A dog was fed 3 pounds of milk and 1 pound of cane sugar the first day, the same the second day, and 2 pounds of milk and 2 pounds of sugar the third day, at the end of which time the pH of the jejunum was 6, of the middle ileum 6, and of the lower or distal ileum 6.7.

In rabbits it was necessary to distinguish between different regions of the intestine, as shown in Table I which gives averages on different feeds.

TABLE I.

pH of Ileum of Rabbits.

Food.	Proximal.	Middle.	Distal.
Lactose and oats	6.5	6.6	6.8
Carrots	6.51	6.65	6.76
" and oats	6.91	7.32	7.66
Oats	7.12	7.34	7.51

The results may possibly show that the more soluble carbohydrate there is in the food, the more acid the intestine may become, yet the data do not warrant any positive statements on the subject. In fact, we began to suspect that the length of the ileum might be a factor, since in long ileums the acidity decreased on the way down.

If the ratio of the length of the ileum to that of the body is taken as an index of the relative length of the former we obtained an index of 8.6 for the cat and 13 for the rabbit, using the same methods of measurement. It is then necessary to test whether the relatively shorter intestine is more acid in all cases as it is in the above comparisons of carnivora and herbivora. In order to compare the same species we used suckling and adult rabbits. The index of the former was 10 and the latter 13. (In man the reverse change occurs, the index for new-born infants being 7.9 and for adults 3.8.) So the young ones would be expected to have a more acid ileum, as was actually found, the average values being 6.4 pH for the proximal and 6.61 for the distal portions in four young rabbits. Since this was even more acid than it was possible to make the ileum of adults by diet, we infer that the milk diet of

the young is not the only factor but that the shorter intestine is associated with more acid contents.

Since we did not find appreciable amounts of ammonia in alkaline intestinal contents, and the original pH of all the food tested was acid, we concluded that the variations in pH must be due to some other factor, such as the absorption of CO_2 as the food moved down the intestine. Long and Fenger[1] determined the pH and CO_2 tension of the intestinal contents of hogs, their average figures being as follows:

Part of ileum.	pH	CO_2 tension in per cent of an atmosphere.
Proximal	6.7	23.6
Middle	6.96	13.0
Distal	7.1	14.0

It therefore appears that the intestinal contents become alkaline because the CO_2 is absorbed faster than it is produced. As the gastric juice mingles with pancreatic secretion in the duodenum, a

TABLE II.

pH of Rabbit's Ileum.

No.	Age.	Proximal.	Middle.	Distal.	Diet.
1	4 weeks.	6.13		6.32	Nursing.
2	4 "	6.32		6.54	"
3	8 "	6.45		6.68	" and eating oats and carrots.
4	8 "	6.73		6.90	" " " " " "
5	Adult.	6.91	7.40	7.80	Oats and occasionally carrots.
6	"	6.84	7.31	7.72	" " " "
7	"	6.8	7.28	7.62	" " " "
8	"	6.93	7.48	7.89	" " " "
9	"	6.70	6.90	7.10	" " " "
10	"	7.40	7.71	8.10	" " " "
11	"	6.81	7.20	7.39	" " " "
12	"	7.50	7.70	7.92	"
13	"	7.00	7.18	7.25	"
14	"	6.97	7.14	7.37	"
15	"	6.81	6.90	7.00	"
16	"	6.62	6.89	7.20	Carrots.
17	"	6.09	6.28	6.34	"
18	"	6.65	6.78	6.94	Water only.

large amount of CO_2 is produced more of which is absorbed the longer the intestine. Bacterial action, however, would partially restore the CO_2 absorbed. (The mineral content of the food does not seem to be a factor in determining the pH since the acidity of the intestinal contents is greater on a diet of carrots that yield a basic ash than on a diet of oats that yield an acid ash.) That the contents of the ileum become less acid on the way down is conclusively shown by Table II.

EFFECT OF DIET ON THE ALKALINE RESERVE OF THE BLOOD.

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The beneficial effects of fruits and vegetables in the diet have been recognized for a long time. Their virtue was at one time attributed to their alkaline ash by Henry C. Sherman and others. Blatherwick[1] has shown that plums, prunes, and cranberries leave acid residues in the body due to the fact that they contain benzoic acid which is not oxidized. It is true, however, that many fruits and vegetables decrease the acidity of the urine, but it has not been proven that the total advantage of them in the diet is due to this cause. In fact the more we learn about vitamines, the more their importance rises and at the same time the importance of ash constituents of food falls.

Our interest in this subject arose from the fact that the Division of Food and Nutrition of the Army paid considerable attention to the acid-base balance of diets and it seemed worth while to show whether or not it was of importance in maintaining the alkaline reserve of blood. By alkaline reserve we mean the bicarbonate concentration of the plasma on the basis of a normal solution of sodium bicarbonate.

The blood was drawn directly from a vein or artery into a tube in which enough dry potassium oxalate had been placed to make 1 mg. per cc. of blood. This was done by measuring the required quantity of 25 per cent potassium oxalate solution into the tube and evaporating it to dryness. In the case of rabbit's blood the quantity was doubled. The blood was drawn and centrifuged with practically no exposure to air or other gas. 1 cc. of plasma was placed in a rotating hydrogen electrode and titrated with 0.1 N HCl under a stream of hydrogen to neutrality (pH = 7).

[1] Blatherwick, N. R., *Arch. Int. Med.*, 1914, xiv, 409.

The titration electrode was an improvement on the one previously described.[2] It is shown in Fig. 1. The electrode vessel was made of a 50 cc. volumetric flask with the neck cut off and the base cemented to a cork pulley-wheel with sealing wax. It was supported on a frame so that the open end would fit over a short piece of tubing attached in the frame and the base was supported by the axle of the pulley. One end of the frame supported a rod holding a micro-burette. After the plasma was placed in the vessel and the latter adjusted in the frame, a plug was carefully introduced through the short section of tubing into the electrode vessel. This plug was made of a short piece of rubber tubing through which passed a bundle of tubes the whole plug being made coherent by means of sealing wax. The bundle of tubes consisted of (1) a glass tube admitting a constant stream of hydrogen, (2) the tip of the burette, (3) a minute rubber tube filled with a saturated solution of KCl and closed at the inner end with a bit of match stick, and (4) a glass tube containing a platinum wire. The glass was fused around the platimun wire at the inner end and the outer end of the wire ended in a loop to be connected with the potentiometer. The inner end of the platinum wire had been dipped in melted gold, and before each titration it was cleaned by heating in a flame and plated with iridium by electrolyzing a strong solution (about 50 per cent) of iridium chloride with a 2 volt current for a few seconds, using another platinum wire as anode.

Since an inexhaustible supply of pure hydrogen is necessary for the continued success of the method, all previous sources of hydrogen were discarded and the hydrogen generator shown in Fig. 2 was constructed. Fig. 2 is somewhat schematic and shows only one of the battery of two electrolytic cells. Each cell was made of a beaker into which was inverted a funnel raised from the bottom by means of a piece of glass rod. Two rings of No. 10 nickel wire were made a trifle smaller than the large opening of the funnel and placed, one inside and one outside the lip of the funnel and were continuous with two upright pieces of the same wire projecting out of the cell. A mixture of 30 gm. of KOH and 100 cc. of H_2O was poured into the cell until the nickel rings were covered.

[2] McClendon, J. F., *J. Biol. Chem.*, 1918, xxxiii, 19.

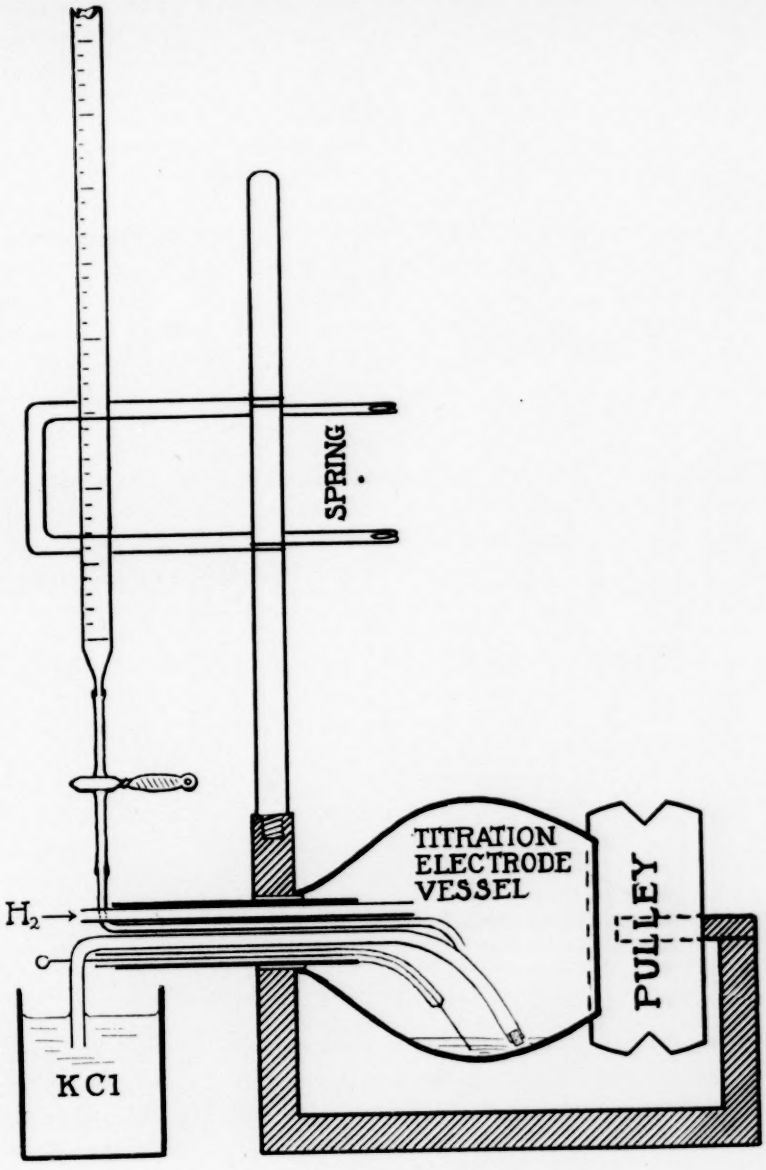

FIG. 1.

The electric current passed from one nickel ring around the lip of the funnel to the other ring with minimal heating of the electrolyte. Hydrogen was generated inside the funnel and passed up the stem and into a thick-walled rubber tube. The nickel wire

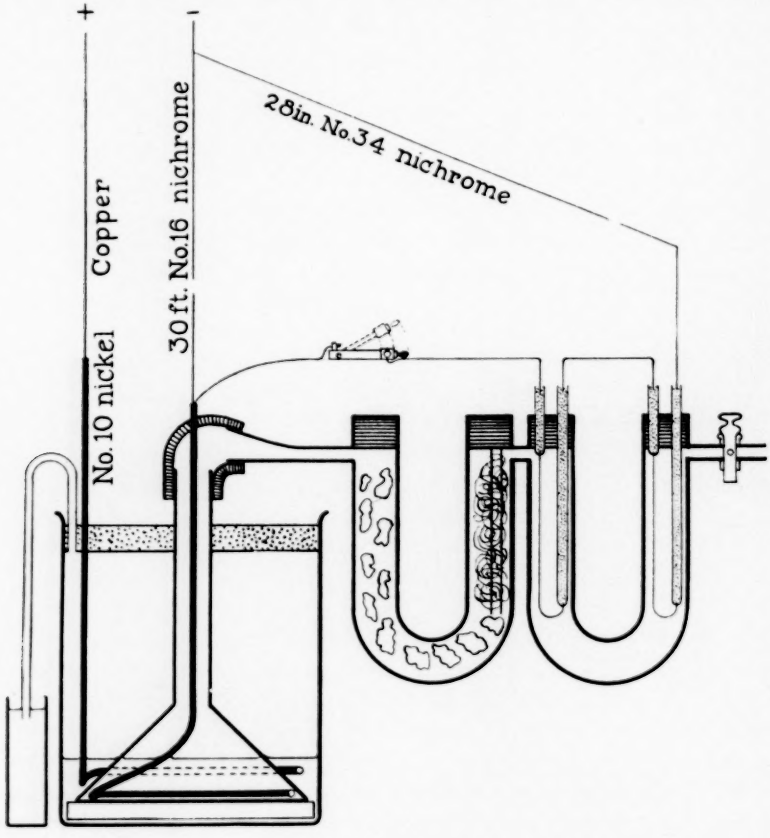

Fig. 2.

connected with the inner ring was sharpened and stuck through the rubber tube so as to connect with the source of current. The above description completes the essential parts of the generator and the additions were merely for regulating the current and pressure and removing the oxygen.

The pressure was regulated by retarding the escape of oxygen and this was done by closing the beaker with a sheet of metal covered with a thick layer of sealing wax. This cover was pierced by three openings, one for the funnel stem, one for the outer nickel wire, and one for a tube leading the oxygen to a trap filled with water to regulate the pressure.

The hydrogen passed from the cell to a U-tube filled with $CaCl_2$ and cotton and then to another U-tube, each arm of which contained a red-hot platinum wire 0.03 mm. in diameter to burn up the oxygen. If the gas is dried before reaching the hot wire, the water formed by combustion will not condense in the U-tube even though it is immersed in cold water to keep it cool (as we do it). The gas is finally passed through a wash bottle filled with 1 per cent NaCl to moisten it, and a spiral copper tube to bring it to the temperature of the room and then into the electrode.

The source of current was 110 volt direct current and the two cells were connected in series. The apparatus was connected to the main current by means of 30 feet of No. 16 nichrome wire stretched free in the air. Part of the current which passed the cells was then shunted off and used to heat the platinum wires. The resistance of the 6 inches of platinum wire was not enough so it was increased by means of 28 inches of No. 34 nichrome wire. When the current was turned off from the cells the platinum wires could not be heated. On first starting the apparatus with air in the U-tubes, an explosion would occur that would break the fine platinum wire, and in order to avoid this, a switch was placed in the shunt so that the air could be washed out with hydrogen before the platinum wires were heated. 20 amperes of current passed through each cell and about 5 cc. of H_2 per second were produced.

The handling of the fine platinum wire requires some care. Two glass tubes were passed through each rubber stopper of the U-tube and pieces of larger platinum wire fused in their lower ends. A little gold bead was fused on the end of each wire by touching it in a flame with a gold wire. The 0.03 mm. platinum wire was laid across the gold bead and caused to adhere by reheating it for an instant, the process repeated for the other gold bead, and then the excess of the fine platinum wire was cut off. The glass tubes were filled with mercury and copper wires carrying the current dipped in them.

In making the titrations, if enough plasma was available for triplicate determinations, the first titration was done roughly and usually overran the point of neutrality. The amount overrun was calculated from the titration curve, Fig. 3, and a second titration made by dropping in all the acid at once. Then a third titration duplicated the second. Although the titration curve in Fig. 3 is from one plasma only and may differ somewhat from other plasmas, it is very useful when applied in this way.

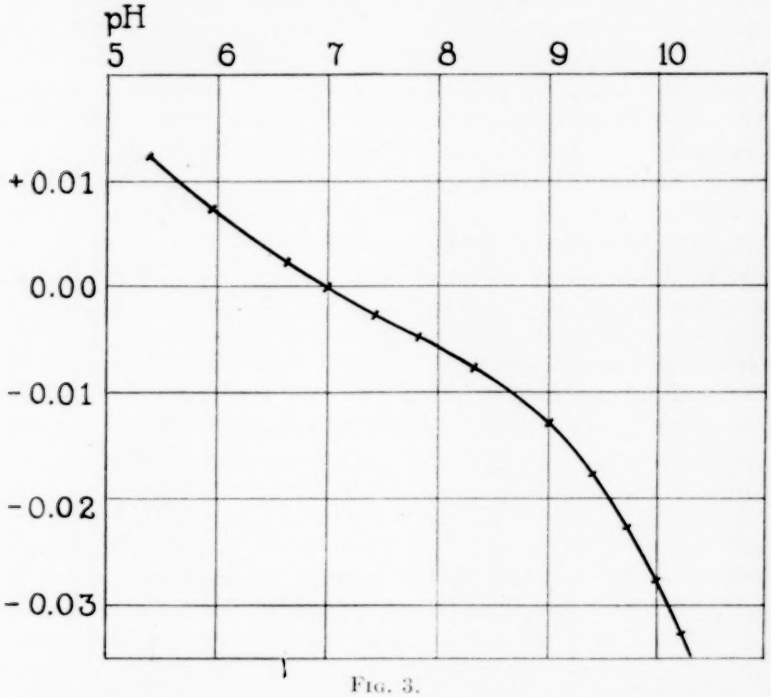

FIG. 3.

Data on the acidity or alkalinity of the ash of foods were taken from Sherman.[3] Protein foods leave an acid ash due to oxidation of sulfur and phosphorus and hence meat, eggs, and all foods made of cereals are called "acid-forming." All vegetable foods except cereals contain salts of organic acids and mineral bases that yield alkaline ashes in excess of the acids from the proteins of the same

[3] Sherman, H. C., Food products, New York, 1915.

foods. Butter and sugar are practically neutral and milk slightly "base-forming."

The following determinations were made on a man of 75 kilos (L. M.). L. M. was placed on an acid-forming diet for 3 days, then a mixed diet for 3 days, and then a base-forming diet for 3 days. Table I gives the results of the determinations. The other data besides the alkaline reserve are included as they might be thought to indicate something in regard to the food intake and metabolism, but except for the total acidity of the urine they were not planned for this paper. The collection of urine was commenced about 6 hours after the first meal and when blood was drawn on a certain 24 hour period, it was drawn at the end of the period. The alkaline reserve was 0.0335 before beginning the experiment.

TABLE I.

Date.	24 hour urine.			Blood sample.		
	Volume.	Titratable acidity.	Total N.	Alkaline reserve.	Amino N per 100 cc.	
1918	*cc.*	*cc. 0.1 N*	*gm.*	*N*	*mg.*	
May 8	1,112	436	15.4			Acid-forming diet.
" 9	1,200	407	14.9			
" 10	925	417	12.3	0.0335	4.3	
" 15	1,125	163	7.3	0.0355	0.7	Base-forming diet.
" 16	1,360	241	8.8			
" 17	1,375	352	9.7	0.0335	1.6	

It may be seen from Table I that the alkaline reserve was not changed by the acid-forming diet from the normal value for this individual (0.0335 N). After 24 hours of base-forming diet the alkaline reserve increased possibly a little more than the limits of error of the method and then went back to normal at the end of 3 days on the base-forming diet. This may have been due to ammonia formation.

Hasselbalch[4] made some determinations on the CO_2 tension of the alveolar air on acid-forming and base-forming diets and found small differences which if they exceed the limits of error indicate

[4] Hasselbalch, K. A., *Biochem. Z.*, 1912, xlvi, 403.

that the alkaline reserve may be 0.003 N greater on a base-forming than on an acid-forming diet or during fasting. Similar determinations by Blatherwick[5] show differences of 0.002 N. The method of reduction of CO_2 tensions to alkaline reserve values has already been described.[6]

Van Slyke, Cullen, and Stillman[7] found that the alkaline reserve of the blood rises during gastric digestion, due to the secretion of HCl from the blood to the stomach. We drew blood at the same stage of gastric digestion (about half hour after luncheon) in each case in the hope of avoiding error due to complication from this source.

In order to use a more easily controlled diet, experiments were made on dogs. Blood was taken from the carotid arteries of four dogs and titrated. The results are given in Table II.

The fifth dog was placed in a cage March 4 and fed on 350 gm. of raw lean beef per day for 2 months and blood drawn from an ear vein at intervals and from the carotid at the end of the experiment. The blood drawn on normal diet and some specimens taken later were discarded due to clotting or getting air in the tube, but samples were correctly drawn on March 13 and May 4 and titrated 0.029 and 0.03 respectively. Since this is the average for the four normal dogs within the errors of the different methods of taking blood, we conclude that it is impossible to lower the bicarbonate concentration or alkaline reserve of dog's blood by a highly acid-forming diet for 2 months.

It was thought that rabbits might be more susceptible to changes in diet since Scott[8] had shown that the alveolar CO_2 of rabbits varied from 4.57 to 6.3 per cent of an atmosphere. Hasselbalch[9] gives the pH of rabbit's blood as 7.33 or very nearly what he got for man with his early technique, and, assuming it is the same as man, the above alveolar CO_2 values would place the alkaline

[5] Blatherwick, N. R., *Arch. Int. Med.*, 1914, xiv, 445.

[6] McClendon, J. F., Shedlov, A., and Thomson, W., *J. Biol. Chem.*, 1917, xxxi, 519.

[7] Van Slyke, D. D., Cullen, G. E., and Stillman, E., *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 184.

[8] Scott, F. H., *J. Physiol.*, 1908, xxxvii, 316.

[9] Hasselbalch, K. A., and Lundsgaard, C., *Skand. Arch. Physiol.*, 1912, xxvii, 31.

reserve at 0.024 to 0.032 N. Kurijama[10] found that food changed the alkaline reserve of rabbit's blood. His figures in our units would be 0.026 N on base-forming diet and 0.021 on acid-forming diet. His results are complicated, however, by the fact that he used oats for the latter diet and oats contain no antiscorbutic substance and scurvy has been considered to be associated with acidosis. We wished to avoid this by adding fresh sprouted barley to the oat diet, as sprouted barley cures scurvy in guinea pigs. The rabbits were bled from the carotid artery under ether. The results are shown in Table III.

TABLE II.

Dog No.	Alkaline reserve.
	N
1	0.03
2	0.025
3	0.03
4	0.03

TABLE III.

Rabbit No.	Diet.	Alkaline reserve.	
		N	
1	Oats and sprouting barley.....	0.0145	
2	" " " "	0.0175	Acid-forming diet.
3	" " " "	0.007	
4	Fasted (water).....	0.0155	
5	Carrots and hay.....	0.023	
6	" (insufficient number).....	0.013	Base-forming diet.
7	" and cabbage.....	0.021	

These rabbits were kept on the diet 1 week except Nos. 1 and 5 which received the diet 9 days and No. 4 which received water but no food for 6 days. The results are not harmonious and the variation may be due to the different amounts of antiscorbutic substances the rabbits received in the period previous to the experiment as well as during the experiment and to partial starvation; *i.e.*, insufficient quantity of food at certain intervals. Thus

[10] Kurijama, S., *J. Biol. Chem.*, 1918, xxxiii, 215.

No. 4 which fasted showed as low an alkaline reserve as those on acid-forming diet and this is in harmony with the view that fasting induces acidosis or that the rabbit was living on its muscle tissue (as well as fat). It is evident, however, that the alkaline reserve is influenced by diet.

CONCLUSIONS.

The alkaline reserve of man and dog is remarkably resistant to influence of diet, whereas the rabbit is susceptible to the effects of diet and fasting. There is no foundation for the view that the alkaline reserve of man is endangered by acid-forming diets but such diets as usually eaten are deficient in antiscorbutic substances.

NOTE ON THE ULTRAMICROSCOPY OF EGG ALBUMIN.

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The more that is known of the physical chemistry of proteins, the less they appear to resemble the suspension colloids. Sörensen[1] has studied egg albumin from the standpoint of an electrolyte, and considers the molecular weight to be about 3,400, and the pH of the lowest osmotic pressure 4 to 4.4, of the lowest solubility 4.58 (in presence of ammonium sulfate), and of the isoelectric point 4.8. He has shown that it can be purified by recrystallization and that the crystals probably consist of 2 albumin molecules, 3 molecules of sulfuric acid, and 830 water molecules.

If proteins exist in true solution we would expect ultramicroscopic particles to be absent or very numerous, depending on whether the individual molecules could be seen or not. We recrystallized egg albumin three times by Sörensen's method and made a saturated solution of the third crystals in distilled water. The pH of this solution was about 4.2 and the ultramicroscope showed only an occasional submicron. On titrating it to pH = 4.8 in a hydrogen electrode by means of 0.1 N NaOH and then re-examining it with the ultramicroscope we found a slight increase in the number of submicrons, but this may be due to the fact (also observed by Sörensen) that a precipitate forms very slowly in such solutions on long standing, and we suppose the submicrons may be the first indication of a change resulting in precipitation or denaturation of the albumin. We believe that in the absence of denatured albumin there would be no submicrons present. The presence of the Tyndall effect can hardly be taken as proving the presence of colloid particles, since it has been observed in solutions of sugars that are not colloids in the usual sense of the term. It seems unfortunate to us that clear solutions of proteins should be classed with suspensoids under the term "colloids."

[1] Sörensön, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.

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